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**PROCEEDINGS OF THE 5th ANNUAL CONFERENCE
ON ENVIRONMENTAL TOXICOLOGY**

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AEROSPACE MEDICAL DIVISION
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FOR THE COMMANDER



ANTHONY A. THOMAS, M.D.
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FOREWORD

The Fifth Conference on Environmental Toxicology was held in Fairborn, Ohio on 24, 25, and 26 September 1974. Sponsor was the University of California, Irvine under the terms of Contract F33615-73-C-4059 with the Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio. Arrangements were made by the Toxic Hazards Research Unit of the University of California, Irvine, and the papers presented at this Conference by personnel of the University of California represent research conducted under the cited contract. B. Dwight Culver, M. D., Associate Clinical Professor, Department of Community and Environmental Medicine, University of California, Irvine, Irvine, California served as Conference Chairman, and Mrs. Lois Doncaster, University of California, served as Conference Coordinator.

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OPENING ADDRESS

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Ladies and gentlemen, it is a pleasure to be here with you at this Fifth Conference. I have had the pleasure of attending several of these conferences in the past, and I can remember the times in which we have called them something else fitting the toxicology needs of the particular time period. It is of interest to note that the scope of your activity has increased considerably, and now includes the long range impact of chemical operations. This is of great significance to the Air Force, of course, and probably the result of some of the newer Federal regulations.

The program of the Conference also underscores additional concern in that you have dedicated an entire session to the topic of carcinogenesis. This is, again, of great concern to us. I was asked when I came here to describe to you the Air Force's real commitment to toxicology. I think the Air Force commitment in a certain sense is to protect man in his environment, particularly in this day and age when the Air Force is at the forefront of materials technology and propellants technology. We must be more alert than ever before to the toxic properties of new materials and new propellants.

I think we must understand the toxicological properties so that we can protect not only the Air Force man - the guy that supports the system - perhaps the man that flies in the system - but in addition to that the person who is outside the system. This is the problem that we get involved in regarding the long term effects - environmental effects - of these toxic products. More important than that is our responsibility within the Air Force to interpret your work so that the operator, the commander of a base, the man that is using these particular materials, understands the restrictions that we place on their use.

I think our record to date in the Air Force has been quite good and we must keep it that way. We have had a number of more exotic materials, I guess you could call them, in the past few years that have not been of great concern in regard to long term effects on our own people and, in fact, people out of the confines of our own bases. We must now worry about long term effects. In order to get the operator of a base to understand the use of some of the exotic materials, we must provide him with criteria for its use. Today these criteria must be provided within the intent of Federal regulations.

The other matter is the fact that if we place undue restrictions on the use of some of these toxic materials, or potentially toxic materials, then we can in effect influence Air Force operations and influence them probably in an untoward fashion. This is why our toxicology program must provide information that enables us to develop realistic criteria. Prejudgment of these same materials could occur from other sources that would be unduly restrictive. Undue restrictions might be placed on launch profiles of certain types of missiles that are used in the Air Force, especially some of the satellite missiles that are used in which we have to get into a certain window for the launch. If we place undue restraints on these people, we then limit the time of operations and the time that these missiles can be launched, which is very significant for some satellites.

Well, that is the Air Force commitment. I think really what was asked was, "What is the Air Force commitment as far as resources are concerned?" I can put that very simply - it's about seven and one-half million dollars of facilities and around two million dollars a year. This is a significant amount that has enabled us to meet the past criteria. It is perhaps not enough - it is perhaps not as much as we would like to have - and I'll address that issue in just a minute - on how we get more in this important area.

What is our future commitment? Of course, our future commitment is to do the same thing. It is to apply these same resources and, if the need requires it, to apply more resources than we presently have. This brings up something that is significant for you. What is your responsibility? What are our responsibilities as individuals? This is the area that becomes of increasing concern to me in these days of lower budgets, limited resources, and perhaps a somewhat endangered economy, as to how we perform something in a preventive fashion with limited resources.

So I think it is part of your responsibility as well as mine to keep at the forefront of what we are doing in a preventive way, to protect man. Let me explain that just a little more. In the reduced budget area, and in the reduced resources area, the one concern in any medical or associated field becomes a health care delivery system and that overwhelms us. It overwhelms us in that it eats up all the resources that are the immediate need. There becomes an attempt to cut back on everything else unless it is of immediate need - something that might not be noticed in the near future. For that reason, it is up to you and to me to keep influencing people.

Although health care delivery systems need the resources, we dare not neglect important research if it involves the future health of the individual, and might in effect extend those health care resources some time in the future in an unwarranted fashion. So that is part of your commitment, and that is part of my commitment, in a sense that cannot be put into dollars. Perhaps it gets us to go out and talk to people and try to convince them that we cannot relegate research in toxicology to a level that keeps us from being in the forefront in the years to come.

This conference has always been a most important means of communication. I have had the opportunity to observe some perfectly delightful, wonderful, heated exchanges of information. This type of exchange about some of the important work can only do one thing, and that is lead everyone to learn more and to develop new opinions. So it is a good interchange of information. The forums that are provided probably give us the best results when they allow everyone to address the issue and voice opinions regardless of how critical they might be.

I wish you the best of success for one of the finest meetings.

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SESSION I

ENVIRONMENTAL QUALITY: EVALUATION AND RESEARCH

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DEVELOPMENT OF LABORATORY MODEL ECOSYSTEMS AS EARLY WARNING ELEMENTS OF ENVIRONMENTAL POLLUTION

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INTRODUCTION

Problems of environmental pollution with toxic substances, singly or in combination, have greatly intensified during the past three decades. In the 1940's we were told that the "solution to pollution was dilution" and we were confident that the capacity of our environment for absorption, dilution, and detoxication was infinite. Today we are deeply concerned about the parts per trillion levels of pollution of the Great Lakes with DDT, dieldrin, PCB's, Hg etc. and their 10^5 to 10^7 biomagnification in fish and consequent entry into terrestrial food chains and into the human diet (E. P. A., 1972). Trace contamination of agricultural soil at 0.1 - 1 ppm with dieldrin, the epoxidation product of the insecticide aldrin, has been picked up in cotton and soybeans, and introduced by plant oil sludges used as feed supplements into the diets of as many as 25 million chickens which have been destroyed because of the illegal residues of potent carcinogen that they contain (Randal, 1974). Traces of lead from tetraethyl lead in gasoline and cadmium from rubber chemicals in automobile tires have polluted our cities and the verges of our highways until our streets are literally paved with these metals and our house dust is unsafe to breathe (Ewing and Pearson, 1974).

Most of the source of our concern about environmental pollution by trace substances relates to the inventiveness of the chemical industry and to its exponential growth. The first edition of the Merck Index (1889), "containing a summary of whatever chemical products are today adjudged as being useful in either medicine or technology," listed about 828 chemicals, the eighth edition (1968) more than 10,000. The "Toxic Substances List" NIOSH, (1973) catalogues 25,043 potentially hazardous chemicals. Quantitatively, organic chemical production in the United States has risen from 10 billion pounds in 1943 to 140 billion pounds in 1972. The rate of growth is exponential increasing at an average of about 9% per year (doubling time 8 years) and the 1972 production was equivalent to 676 pounds per capita.

These chemicals have almost endless uses as intermediates in synthesis, fuel and fuel additives, plastics, elastomers, plasticizers, brighteners, heat-transfer agents, flame retardants, rubber chemicals, paints, lacquers, solvents, fibers, detergents, insecticides, fungicides, herbicides, bactericides, coccidiostats, growth promoting agents, and pharmaceuticals. They enter the environment accidentally from industrial effluents, household applications, or as by-products of transportation; or purposefully from application of pesticides, protective coatings, and preservatives. It is estimated that from 500 to 700 new chemicals are produced each year on a scale large enough so that significant traces of them enter directly into the environment (Lee, 1964). As environmental micropollutants, these chemicals may be harmless, toxic, carcinogenic, mutagenic, or teratogenic. The effects may require many years to determine in the human population, e.g. the recent indictment of vinyl chloride (U. S. production 5.35×10^9 lb. in 1973) as a human carcinogen (CDC, 1974), the discovery of phthalate ester plasticizers (U. S. production 1×10^9 lb.) as teratogens (Singh et al., 1973), the implication of PCB residues in Lake Michigan fish as the cause of sterility of domestic mink (Ringer et al., 1972), and of atmospheric manganese emissions from mining, iron production, and battery production (U. S. manganese consumption 1.55×10^9 lb. in 1968) in producing manganism, a neurological disorder closely resembling parkinsonism (NAS, 1973). The most alarming implication of trace element pollution is a recent World Health Organization estimate that 85% of all human cancers have an environmental cause (C & E News, 1974).

These astronomical figures together with exponential growth of the U.S. and world chemical industries makes it easy to appreciate the necessity for a "Toxic Substances Act" such as that now under consideration by Congress. The proposed Act will require the manufacturer or user to provide proof of the environmental safety of the various "toxic substances." The proof will require the employment of various "early warning" techniques and it appears that some sort of model ecosystem which can demonstrate the degree of biodegradation and possible biomagnification and food chain transfer of "toxic substances" will become an important component of such "early warning" evaluations.

DEVELOPMENT OF LABORATORY MODEL ECOSYSTEM

Our laboratory became interested in 1966 in the development of a simple laboratory model ecosystem under a grant from the Herman Frasch Foundation, American Chemical Society. Our idea was to use radiotracer methodology to follow the movement and degradation of pesticides through a series of food chain transformations and to develop information about:

- 1) toxic effects of pesticide and its degradation products in organisms from at least 5 phyla.
- 2) degradative pathways of pesticide in the environment.
- 3) bioaccumulation of pesticide and its degradation products.
- 4) biodegradability of pesticide and its degradation products.

It was our purpose to obtain all of this information in a simple, inexpensive laboratory system which could be used in environmental toxicology in a manner analogous to the use of the rat in industrial toxicology.

Initially we chose to model the application of the pesticide to a farm, orchard, or forest, and to follow by the radiotracer technique the movement and fate of the compound into a lake. Real world analogues could range from application of a pesticide to a cornfield and its transfer into a farm pond or the application of DDT to elm trees in the Chicago area for the control of the bark beetle vectors of Dutch elm disease and the consequent dispersal into Lake Michigan. In the latter case, it is well known that DDT in the lake at 0.000002 ppm has become biomagnified into fish up to 10 - 20 ppm or 10^7 -fold (EPA, 1972; Harrison, 1970).

The methodology of this system has been described in detail (Metcalf et al., 1971, 1974a, 1974b). Very briefly it is housed in a 10 x 10 x 20" glass aquarium containing a sloping shelf of washed white sand. The lower portion is covered with 7 liters of standard reference water which forms a "lake" in which are grown plankton, alga (Oedogonium carciacum), Daphnia magna, mosquito larva (Culex pipiens), snail (Physa sp), and fish Gambusia affinis) which provide the test organisms and food chain components of the system. On the flattened terrestrial portion are grown Sorghum vulgare plants which provide the "farm" portion of the ecosystem.

The aerated aquarium is partially covered with a plexiglas cover to retard evaporation and is placed in an environmental plant growth chamber at 80 F with a 12-hour simulated daylight of 5000 foot candles.

Several different protocols of operation of this model ecosystem have been developed: (1) the typical pesticide evaluation involves quantitative application of 1 to 5 mg (0.2 to 1 lb./A. equivalent) of the radiolabeled pesticide (^{14}C , ^3H , ^{32}P , ^{35}S) from acetone solution to the leaves of the Sorghum. The treated plants are then eaten by the salt marsh caterpillar Estigmene acrea and this serves as the dispersing agent for the labeled micropollutant. (2) The radiolabeled micropollutant is injected by micro-pipette as an acetone

solution into the sand of the terrestrial portion to simulate a pre-emergent herbicide or is applied to Sorghum seeds to simulate seed treatment with insecticide or fungicide. (3) The radiolabeled micropollutant is added directly to the water portion to simulate an aquatic effluent pollutant. (4) The micropollutant is incorporated directly into standard soil types which is added to the sand to simulate such problems as lead pollution along the highway, addition of sewage sludge to crop land, or soil herbicide or insecticide adsorption and erosion. In place of radiolabeling, atomic absorption spectrometry or neutron activation analysis can be used for the ultimate analysis.

Following any of these methods of application, the model ecosystem units are left for 33 days and at the end of the experiment the organisms are sampled and counted for total radioactivity, extracted with organic solvents and the extracts separated into chemical constituents by thin layer chromatography (TLC) and radioautography, followed by liquid scintillation counting to quantitate the results. From this workup we obtain (a) the ecological magnification (E. M.) or ppm of parent compound (or a particular degradation product) in organism/ppm in water; (b) the biodegradability index (B. I.) or ppm of polar radioactivity in organism/ppm of non-polar radioactivity (from TLC plates), (c) the degradative pathways from determination of the chemical nature of degradative products by cochromatography with known model compounds, mass spectrometry, etc, and (d) the unextractable or totally metabolized radioactivity.

Applications of Terrestrial Aquatic Model Ecosystem

We have employed the model ecosystem as described above in several major ways.

1. Evaluation of pesticides intended for agricultural use in Illinois.

In cooperation with the Illinois Natural History Survey we are routinely screening all pesticides intended for use in Illinois and to date have completed evaluation on about 60 pesticides. Illinois corn and soybean culture is one of the heaviest users of pesticides in the world and the magnitude of the task is indicated by the introduction under experimental permit of 10 new compounds in 1974. Essentially nothing is known about their environmental toxicology and fate. Unfortunately this is also the situation with many of the older well established pesticides. Model ecosystem studies of a number of organochlorine insecticides have been reported (Metcalf et al., 1973). As we increase the number and range of pesticides evaluated we will be able to project rather accurately the environmental effects to be expected from compounds with E. M. values and B. I. values in particular ranges.

2. Evaluation of insecticides intended for vector control by WHO.

The problems of vector resistance to the older insecticides such as DDT and lindane, and new and important vector-control programs, e. g. the \$100 million onchocerciasis control program in Mali and Upper Volta of Africa (WHO, 1973), have created an urgent demand for new types of insecticides. There is an important need to demonstrate their safety and biodegradability, especially for compounds applied directly to water for control of the blackfly larva Simulium damnosum, vector of Onchocerca volvulus.

WHO has requested our International Collaborative Center for Insecticides at the University of Illinois to undertake model ecosystem evaluation of all new pesticides intended for field experimentation in vector control. To date we have evaluated approximately 20 insecticides, including methoxychlor and other DDT analogues, organophosphorus and carbamate insecticides, and insect growth regulators. The evaluation of chlorpyrifos (Dursban®) and its methyl analogue is illustrative. Chlorpyrifos (OMS-971) is one of the most effective larvicides found in the WHO "Programme for the Evaluation and Testing of New Insecticides" (WHO/VBC/71, 1971). However, it is undesirably toxic to humans (rat oral LD₅₀ 135 mg/kg) and its O, O-dimethyl analogue, methyl chlorpyrifos (OMS-1155), which is nearly as effective as a larvicide and much safer (rat oral LD₅₀ 1000 mg/kg), was also considered for use (Quellenec, 1972). A decisive factor was the relative persistence and biodegradability of the two compounds. With the excellent cooperation of the Dow Chemical Company, ¹⁴C-radiolabeled chlorpyrifos and methyl chlorpyrifos were compared in model ecosystem evaluations (Metcalf, 1974). The results showed that methyl chlorpyrifos, E.M. 95, B. I. 3.95, was much more biodegradable and less biomagnified than chlorpyrifos, E.M. 314, B. I. 1.02.

3. Development of Principles of Biodegradability and New Biodegradable Insecticides. Our laboratory, sponsored by a grant from the Rockefeller Foundation for the "Development of Novel, Non-Persistent Insecticidal Compounds" has been studying the effects of incorporating degradophores or groups serving as substrates for the microsomal oxidase enzymes, into the basic structure of the DDT type molecule. Quantitative data on E.M. and B.I. from laboratory model ecosystem studies for more than 15 DDT analogues altered in both aromatic and aliphatic positions has been essential in demonstrating biodegradability and exploring degradative pathways (Metcalf et al., 1972).

4. Evaluation of the Micropollutant Properties of Toxic Substances.

As new alarms are raised about the distribution and fate of newly discovered micropollutants, the model ecosystem approach has repeatedly demonstrated its value in producing specific data about bioconcentration, food chain transfer, degradation pathways, and toxicity levels to various organisms. During the past 5 years we have conducted model ecosystem studies of such new pollution problems as mirex, hexachlorobenzene (Metcalf et al., 1973a) TCDD, dioctyl phthalate (DOP) plasticizer (Metcalf et al., 1973b), and the polychlorinated biphenyls (PCB's) (Metcalf et al., 1974). The PCB studies were conducted with pure ^{14}C -radiolabeled tri-, tetra-, and pentachlorophenyls and demonstrated the pronounced decrease in degradative pathways which resulted from an increasing number of chlorine atoms. Thus the biomass recovery with trichlorobiphenyl was 0.45%, with tetrachlorobiphenyl was 8.7%, and with pentachlorobiphenyl was 57.2%. No evidence could be detected of the environmental conversion of DDE to PCB type compounds.

PROLIFERATION OF THE MODEL ECOSYSTEM CONCEPT

The wealth of information readily available from the radiotracer investigations of micropollutants in small model ecosystems and the urgent demands for information about the environmental behavior and fate of chemical contaminants has resulted in extension of the model system concept far beyond our original proposal. Today we have investigations in progress with at least four modifications of the original system, dealing with pesticides, plasticizers, veterinary drugs, carcinogens, food additives, industrial chemicals, trace metals, and organic impurities.

Model Aquatic Ecosystem

The effluents from industrial manufacturing contain thousands of organic chemicals whose environmental fate requires investigation. These ultimately find their way through sanitary sewers, dumping, etc. into the aquatic environment. The model aquatic ecosystem was devised to measure bioaccumulation and food chain movement of such contaminants using as components the same organisms as used in the terrestrial-aquatic ecosystem. The system is totally enclosed in a 2-liter round-bottom 3-neck flask fitted with traps for organic vapors and CO_2 . Thus it is possible to investigate relatively volatile compounds, including vinyl chloride, and to obtain quantitative data on the total breakdown and distribution of the contaminant. Using this system, we have studied the environmental fate of a series of simple benzene derivatives: aniline, anisole, benzoic acid, chlorobenzene, nitrobenzene, and phthalic anhydride, and the specialty chemicals hexachlorobenzene, pentachlorophenol, 2,6-diethylaniline and 3,5,6-trichloro-2-pyridinol

(Metcalf and Lu, 1973; Lu, 1974). Quantitative relationships were developed between the intrinsic molecular properties of the contaminants and the biological responses. Water solubility was related to ecological magnification with a correlation constant $r = -0.92$ and the octanol/ H_2O partition constant π to ecological magnification, $r = 0.79$. Considerable attention was given to the comparative degradation pathways in organisms of the several phyla. It was found that electronic properties (sigma constant) for the various benzene derivatives were related to the rate of degradation and loss of the compounds in the organisms, with a correlation constant $r = 0.91$ (Lu and Metcalf, 1975). This work provides a theoretical basis for relating basic molecular characteristics to environmental behavior.

Rice Paddy Model Ecosystem

Rice is the world's most important crop and is grown in peculiarly intimate aquatic-terrestrial environment. Rice culture is extraordinarily intensive and a very large variety of pesticides, herbicides, insecticides, and fungicides are used in rice culture. For example, in Taiwan in 1969 approximately 3 kg of pesticides per ha. were applied to the rice crop, representing 60% of the total pesticide consumption on the island. More than 200 pesticide chemicals are used and the problem is complicated by the multi-cropping system with 2 or 3 crops yearly, and by the importance of fish culture in rice paddies. Highly persistent chemicals such as BHC and pentachlorophenol have seriously affected rice culture in Japan (Goto, 1971). The rice paddy model ecosystem represents only a minor modification of the terrestrial aquatic system, and uses a higher water table with rice, Oryza sativa, grown on sand. A completely screened cover, with removable hatch permits using rice leafhoppers and lepidopterous pests as typical components of the rice-ecosystem. Goldfish, Carassius auratus, are used as representatives of the carp family. Problems currently under investigation include interactions between esterase inhibitors such as the insecticide carbaryl which inhibits the degradation of the rice herbicide propanil, so that injury to rice results when they are used together (Kaufman et al., 1970). Such problems of pesticide interaction are particularly amenable to study in model ecosystems, using combinations of labeling, i. e. with 3H and ^{14}C .

Veterinary Drug Model Ecosystem

This research project (supported by the Food and Drug Administration Contract 74-127) has just begun and is planned to model the administration of veterinary drugs such as coccidiostats, growth hormones, antibiotics, and feed supplements to animals in food lots with consequent loss of the drugs and their metabolic products into the environment. A modification of the terrestrial aquatic model ecosystem in which the drug is administered to baby chicks or white mice caged above the terrestrial "farm" area is being used. Compounds under study initially include diethyl stilbestrol, selenium, and phenothiazine.

Terrestrial Model Ecosystem

This research project, just inaugurated (supported by the Environmental Protection Agency Grant R 803249), involves the application of radio-labeled pesticides to three typical agricultural environments: corn-soil, soybean-soil, and cotton-soil. The "ecosystem" consists of a 5 gallon wide mouthed jar in which 50 plants are grown in vermiculite or soil-vermiculite mixtures with synthetic water. The jars contain typical invertebrate populations of insects, earthworms, slugs, and isopods, and the food chain transfer is completed to the mouse (Microtus) as a general omnivore. Samples are taken at intervals of the soil, the plant, and the air to determine the total fate and balance of the radiolabeled compound under investigation.

CONCLUSIONS

To date we have studied the fate of approximately 100 organic compounds in the several types of model ecosystems described. This has given us a considerable background of data to relate the behavior of the compound in the model system to that in the real environment. We believe that the use of quantitative parameters such as ecological magnification (E.M.) and biodegradability index (B.I.) will enable us to relate any new or potential pollutant to well characterized compounds, and that from such information we can predict areas of probable environmental concern. The use of the model ecosystem technology provides a relatively simple way to measure the environmental toxicology and degradation pathways for new compounds in a variety of organisms. Model ecosystems are invaluable for screening new compounds for environmental persistence and degradation to demonstrate which of many possible derivatives or modifications are least hazardous to environmental quality. Model ecosystems provide the only practical way to study the complex interactions involved in the biological behavior of mixtures of environmental pollutants. Finally, model ecosystem studies are unusually graphic and visually appealing and have considerable teaching value for study of the problems of environmental micropollution.

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ECOSYSTEM MODELING

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INTRODUCTION

Environmental systems are extremely complex and therefore the prediction of system responses to external perturbations is difficult and full of uncertainty. The indirect effects of some materials injected into the environment may trigger reverberations through the ecosystem which ultimately lead to an unexpected state of degradation. Mathematical models provide an excellent means for bringing together the state-of-the-art knowledge from a variety of disciplines into a form which can be readily applied to practical problems. These models are being used more and more to represent the dynamic responses of environmental systems. When a model has been developed to the point where it is thought to actually represent natural phenomena (the physical phenomena occurring in nature), it can be used as a management tool to establish policies for the control of environmental practices.

Models have been used for numerous purposes: to gain additional insight about the mechanisms influencing a particular system, to predict the impact of an expected future disturbance to a system, to evaluate alternative methods for improving existing problems, and as part of optimization programs to determine the most economical method of avoiding or alleviating problems in a particular area. It is important therefore that the ability of a particular model selected to represent a system be suited to the purposes for which the model will ultimately be used.

Environmental models vary considerably in the degree of resolution (refinement) with which they represent the physical world. Low resolution models represent general trends for a few linked dependent variables over a limited set of boundary conditions. High resolution models are much more flexible and may represent the responses of a large number of linked dependent variables for a much wider set of boundary conditions. As the order of resolution of a model increases so does the difficulty and cost of its application. The differential equations become complex, and usually non-linear, and

time consuming numerical techniques are required to obtain solutions. The number of coefficients in the model increases and the estimation of coefficient values from observed data is complicated by the non-linearities in the equations. Relatively large amounts of field and laboratory data must be collected because, obviously, the realism of model responses cannot exceed the degree of accuracy of the data used to validate the model. The development of a model for a particular situation, therefore, requires a great deal of engineering judgment with a trade-off between the practicability and economy of model application and the amount and refinement of information to be provided by the model responses.

MODEL LIMITATIONS

Discrepancies between the observed responses of the natural system and those of a mathematical model are due to three types of errors: conceptual, technical, and observational. Conceptual errors represent the difference between the natural system as we see it and the natural system as it really is. That is, conceptual errors are those simplifying assumptions necessary in order to express the real world as a group of mathematical expressions. For example: the assumption of one dimensionality is a conceptual error.

The lumping of several biological groups into a single model parameter may be a significant conceptual error. More than 1.5 million species of plants and animals have been identified. For practical considerations in most ecosystem models, it is necessary to combine several species of organisms having similar characteristics into a single parameter (constituent) in the model. The model parameter represents a measurable characteristic in the real world. For example, the concentration of chlorophyll *a* may be used in the model to represent the group of all species of phytoplankton in a natural aquatic system. This group may include both blue-green algae (procaryotic cells) and other algae (eucaryotic cells). Blue-greens are considerably different from other algae because they are generally slower growing, are not as desirable a food source for organisms in higher trophic levels, and have the ability to fix nitrogen. Blue-greens are the major nuisance organisms in eutropic aquatic systems. Thus, although chlorophyll *a* may be a good parameter for algal activity, and may be a relatively easy characteristic to measure, it can lead to serious misconceptions about the overall system.

Technical errors are those associated with the techniques employed to solve the mathematical expressions. The mathematical expressions for most modern ecological models are too complex for exact solutions and must be solved by numerical methods (computer solutions). All solutions by numerical techniques are available and some are better for certain types of mathematical expressions than others. It is important that the technique selected in any particular model be suited to the specific equations making up the model.

Observational errors are those associated with the measurement of field data. These include sampling errors as well as analytical errors. Our ability to observe natural phenomena may be limited by our capabilities of measuring particular constituents. Frequently the development of a model is directed toward representing system properties which can be easily subjected to analytical decomposition and quantifiable measurements. Properties which are difficult to measure quantitatively may be excluded from a model even though they may have serious impacts on the system.

Because of the conceptual, technical, and observable limitations encountered in representing ecosystems quantitatively, it is usually not feasible to develop "universal" models with widespread applicability to a variety of situations. Thus, it is important that the user of a model be familiar with the model's limitations. There is still a great deal of art involved in the development and application of mathematical models. Each model should be evaluated in the context of its particular objectives.

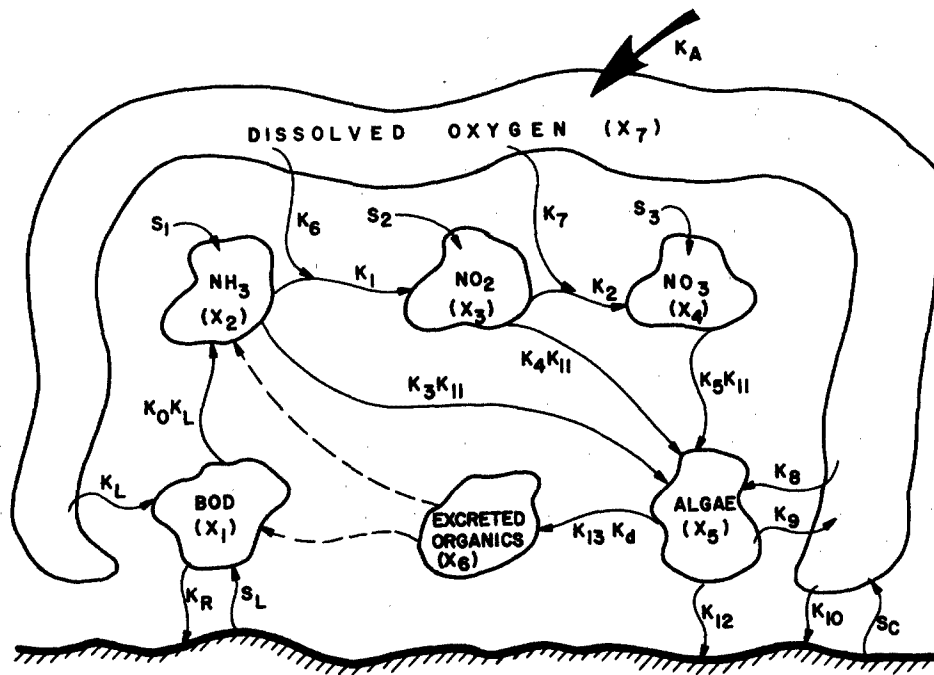
MODEL CONCEPTS

Mathematical modeling can be a powerful tool for the analysis of complex ecosystems when the models are developed with an understanding of the limitations involved. In this section, several approaches for biological systems are presented. Applications to aquatic systems are used as examples.

COMPARTMENT MODEL

Figure 1 is a schematic diagram depicting a compartment model for certain constituents of significance for water quality management. Each compartment represents the concentration of a measurable constituent. Lines connecting compartments represent chemical and biological interactions among compartments. These functional relationships are expressed by the equations in the figure.

Organic material, measured as biochemical oxygen demand (BOD), may be introduced into the model continuously to represent loading from the bottom sediments or other sources (S_L ; mg/liter/day). This material decays at a first-order rate (K_L ; per day) and is adsorbed or deposited on the bottom at a rate K_R (per day). A factor (K_O ; dimensionless) or the decaying material may release ammonia to the water. Ammonia oxidizes at a first-order rate (K_1 ; per day) to nitrite which then oxidizes at a rate K_2 (per day) to nitrate. Ammonia, nitrite, and nitrate may be introduced into the model as continuous loadings by S_1 , S_2 , and S_3 (mg/liter/day) respectively. Nitrogen fixation can be represented by S_4 (per day). Nitrogen may be removed from the system due to benthic algae by assigning negative values to S_1 , S_2 , and S_3 . Ammonia,



$$\frac{dX_1}{dt} + (K_L + K_R + Q)X_1 = S_L + QC_1$$

$$\frac{dX_2}{dt} + (K_1 + K_3 + Q)X_2 = K_0K_LX_1 + S_1 + QC_2$$

$$\frac{dX_3}{dt} + (K_2 + K_4 + Q)X_3 = K_1X_2 + S_2 + QC_3$$

$$\frac{dX_4}{dt} + (K_5 + Q)X_4 = K_2X_3 + S_3 + QC_4$$

$$\frac{dX_5}{dt} + (K_d + K_{12} + Q - S_4)X_5 = K_3K_{11}X_2 + K_4K_{11}X_3 + K_5K_{11}X_4 + QC_5$$

$$\frac{dX_6}{dt} + QX_6 = K_{13}K_dX_5 + C_6Q$$

$$\frac{dX_7}{dt} + (K_A + K_{10} + Q)X_7 = -K_LX_1 - K_6K_1X_2 - K_7K_2X_3 + (K_9 - K_8)X_5 + K_A C_S + S_C + QC_7$$

Figure 1. Schematic diagram showing the model compartments and inter-compartmental transfers and associated equations.

nitrite, and nitrate are taken up by phytoplankton at the rates K_3 , K_4 , and K_5 (per day) respectively and K_{11} is a yield coefficient to convert mg-nitrogen to mg-biomass. Algal biomass may be measured by a convenient parameter (chlorophyll, organic carbon, etc.) as long as consistent units are maintained throughout the model. Algae may be removed from the system by settling (K_{12} ; per day) or death (K_d ; per day). A fraction of the dying algal biomass may be returned to the water as dissolved organics (K_{13} ; mg-BOD/mg-biomass). Dissolved oxygen is added to the system by surface reaeration (K_A ; per day) and by photosynthesis (K_6 ; mg biomass/day) and is removed by BOD (K_L ; per day), nitrification (K_8 and K_7 ; mg O_2 /mg nitrogen oxidized), algal respiration (K_8 ; mg O_2 /mg biomass/day), and diffusion into the sediments (K_{10} ; per day). The addition of oxygen due to benthic algae is represented by S_C (mg O_2 /liter/day).

The equations were developed for a constant volume, completely mixed system such as a chemostat or small well-mixed impoundment. Q is the dilution rate (per day) and represents the flow through the system. The C_i 's are concentrations in the influent stream (Q) and the other coefficients have been defined previously. The equations are solved by an exact technique (Grenney et al., in publication).

As an example of the application of this model, data were taken from McGauhey et al. (1969) who used experimental ponds to observe the impact of various effluents from waste treatment facilities on the growth dynamics of phytoplankton in the oligotrophic water of Lake Tahoe, California. The ponds consisted of fiberglass-coated wooden tanks each about 6.1 m long, 1.2 m wide, and 1.2 m deep with a water depth of 1.1 m and a capacity of about 7960 liters. A Jacuzzi Whirlpool submersible pump was operated continuously in each pond to keep the contents moderately well mixed. During one assay, the continuous influent to ponds 7 and 8 consisted of a 1.0 percent mixture of the effluent from a secondary sewage treatment plant with Lake Tahoe water. The dilution rate (Q) was 0.2 per day giving a residence time of 5 days. Negligible concentrations of nitrate and nitrite were present in the influent and the concentration of ammonia nitrogen ranged between 30 and 300 mg/l as shown in Figure 2(A). Each point represents a grab sample and, therefore, can be used only as a rough estimate of the input to the ponds because of the wide variations in concentrations associated with sewage plant effluents. The points in Figure 2(B) through (D) show the concentrations of ammonia, nitrate, and volatile suspended solids (VSS) as measured in grab samples from the midpoints of the ponds. Dissolved oxygen and BOD were not measured.

The driving function of the model is the ammonia concentration in the sewage plant effluent. The assumed average concentrations used in the model are shown by the solid line in Figure 2(A). Coefficients for the model were estimated by trial and error (Grenney, unpublished paper). Model

responses are shown as solid lines in Figure 2(B)-(D). Some nitrification occurs as indicated by the buildup of nitrate. Both ammonia and nitrate are used as nutrient sources by the phytoplankton (represented by volatile suspended solids). The nitrate and the VSS peaks occur well after the peak in ammonia concentration indicating a lag response in the system.

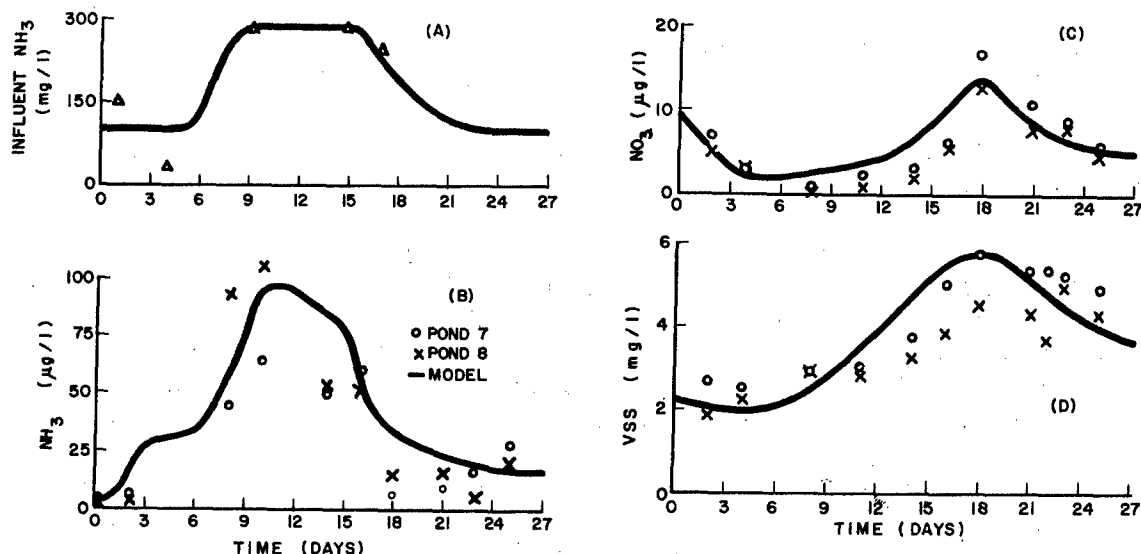


Figure 2. Observed data points and model responses for phytoplankton in a nitrogen limited impoundment (McGauhey et al., 1969).

SUBCOMPARTMENT MODEL

In a compartment model, each compartment represents one of the constituents being modeled in the ecosystem. In a subcompartment model, as defined here, a compartment is divided into subcompartments to represent individual reactions previously composited into a single compartment. The intent is not to model the response of each independent subcompartment, but to provide a more flexible model of the net response of the composite compartment. For example, consider the phytoplankton population represented by the three subcompartments. The model represents a phytoplankton population in a nitrate limited environment with constant optimal light and temperature in Figure 3. We are only interested in the net growth response of the population, but in order to obtain a realistic response we must make provisions for intracellular nutrient pools which have a significant effect on the growth dynamics (Grenney et al., 1973). In the model, the average concentrations of inorganic nitrogen, organic nitrogenous intermediates, and protein within the cells are identified by N_1 , N_2 , and N_3 , respectively, in the model. The environmental concentrations of nitrate and excreted nitrogenous organics are identified by N_I and N_O ,

respectively. The size of the population at any time is measured by the concentration of protein (N_3) in the environment. The coefficient G_1 , G_2 , G_3 , K_4 , K_5 , and K_6 represent reaction rates among subcompartments.

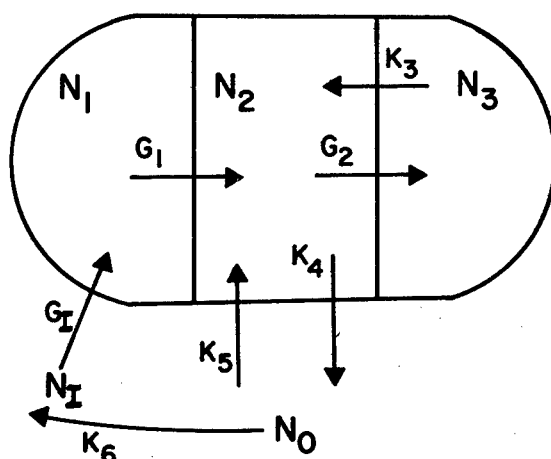


Figure 3. Schematic diagram of a subcompartment model of a phytoplankton population.

Figure 4(A) shows the model response for a batch system with an initial nitrate concentration of $11 \mu\text{g} - \text{N/l}$. It shows several characteristics which are typical of natural populations but are difficult to model using a single compartment. When starved cells are added to a nitrate rich environment, there is a lag phase between the time of adding cells and exponential growth. There is, however, a much shorter lag phase in the uptake of the environmental nitrate (N_1), and this nutrient may be depleted before the maximum population (N_3) is obtained. In order to obtain this realistic response for N_3 , it was necessary to hypothesize the existence of N_1 and N_2 and include them as subcomponents of the model even though no field data were available to substantiate their presence. Figure 4(B) shows the relative proportions of N_1 , N_2 , and N_3 versus time and indicates that substantial amounts of intracellular storage must take place in order for the model to simulate observed characteristics.

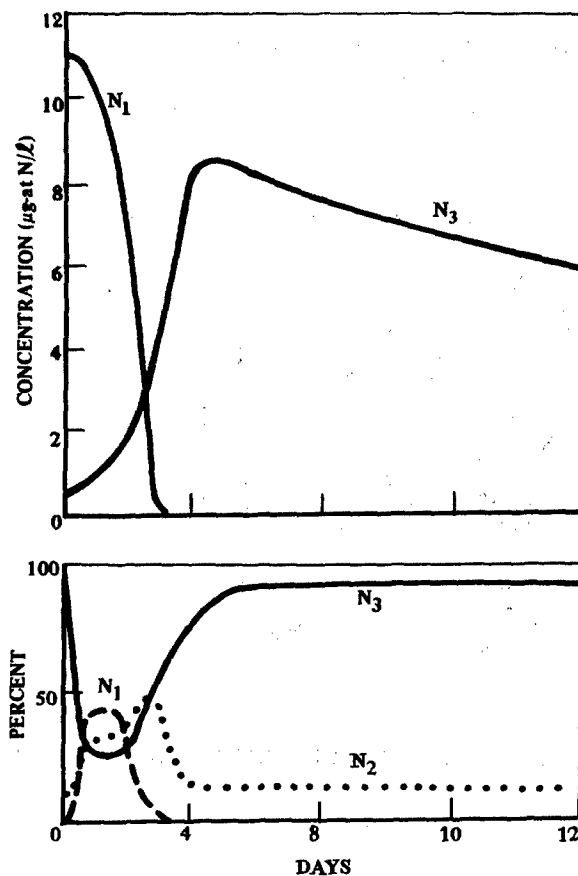


Figure 4. Model responses for a nitrate limited phytoplankton population in a batch system.

COMPONENT MODELING

Component modeling is an approach which has found widespread application in the physical sciences. It provides a methodology for systematically representing the potential, flux, and dissipation of energy within a system. Because biological energetics are important properties of all ecosystems, it is reasonable to assume that this approach may have utility in ecosystem modeling. An energy potential (i.e. voltage in an electrical system) must be measured between two points, one of which is a reference point. Fluxes (i.e. current in an electrical system) represent energy flows and can be measured at a single point. Energy dissipation (i.e. resistance in an electrical system) represents the conversion of energy from a form which is important in the system to one which may be neglected in the system (i.e. from electrical energy to thermal energy in an electrical system).

Hill and Porcella (1974) have presented a thorough discussion of component modeling in biological systems. A component is a mathematical model

of a physical process involving energy flow or transportation. Capacitance is defined as a component which is used to represent the storage of potential. Linear capacitance components can be represented mathematically as:

$$f = C \frac{dN}{dt}$$

where f is the flux, N is potential, t is time, and C is a "capacitance" coefficient. Resistance is defined as a component which is used to represent the dissipation of energy. It is the ratio of potential difference necessary to move a unit of flow through the component in a unit time. A linear resistance can be expressed as:

$$N = Rf$$

where R is the "resistance" coefficient. Connective components describe energy pathways for the interaction of the components within an energy form. A flux junction represents a series pathway; that is, the flux is the same to all connecting components.

The initial component description of the system is best formulated in graphic symbolism. The bond graph representation is convenient and the application of this technique is summarized by Hill and Porcella (1974). The graphic representation is the heart of the component description of the system because the applicability of ensuing analysis is limited by the ability of the investigator to represent his conceptual model of the process in graphic form.

Figure 5(A) is a bond diagram representing a simple conceptual model of the growth and death of a batch culture of organisms. In the figure, P is a potential source component representing the potential energy for growth stored in the environment. N_I is the potential associated with the source. C_3 is a capacitance component representing the storage of biological energy in the living organism. N_3 is the potential associated with this component. R_2 is a resistance component representing the dissipation of biological energy due to synthesis and cell maintenance. N_R is the potential associated with this component. These components are connected in series by a flux junction, JF . Assuming a batch system with a limited potential source which may be expressed as a first order reaction, the model can be expressed mathematically as follows:

$$\frac{dN_I}{dt} = -\beta_1 N_I \quad (1)$$

$$\frac{dN_3}{dt} = \frac{N_I - N_3}{R_2 C_2} \quad (2)$$

where β_1 is first order coefficient. The solution to the system of equations is:

$$N_I = \hat{N}_I e^{-\beta_1 t} \quad (3)$$

$$N_3 = \frac{\hat{N}_I}{1 - R_2 C_3 \beta_1} \left[e^{-\beta_1 t} - e^{-t/R_2 C_3} \right] + \hat{N}_3 e^{-t/R_2 C_3} \quad (4)$$

where \hat{N}_I and \hat{N}_3 represent initial conditions.

Coefficient values were selected so that the initial conditions and the peak biological potential (N_3) would be the same relative scale as concentrations represented by the subcompartment model in Figure 4. The model response is shown in Figure 5(B). It can be seen by comparing Figures 4 and 5 that the forms of the model responses are substantially different for the two techniques.

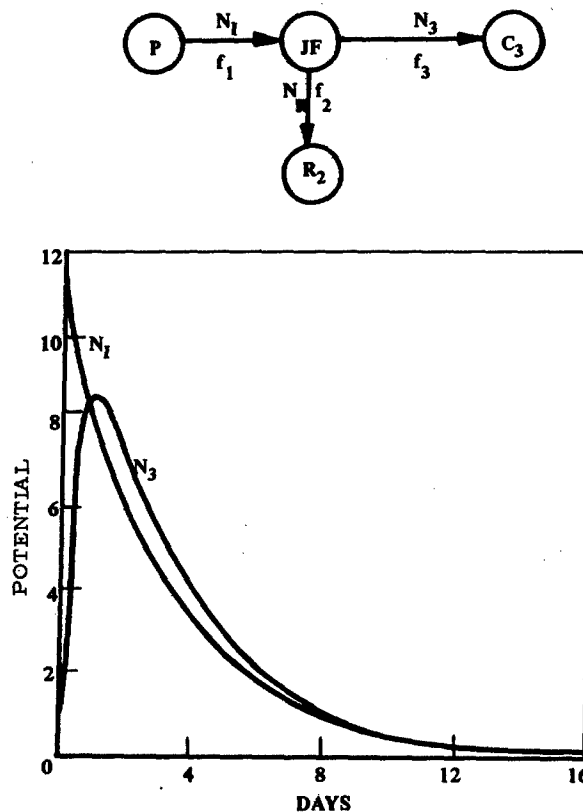


Figure 5. Bond diagram and model responses for a component model.

MODEL APPLICATION

The conceptual models discussed in the previous section were developed for systems in which exogenous influences (external environmental factors) were minimized. The systems were studied at constant temperature and light in uniformly mixed environments. However, for realistic field applications, these biochemical models must be included as parts of larger models of the comprehensive system structure. Examples of methods for representing spatial distributions and variations in temperature, light, and turbulent mixing are presented in this section. The multicompartment model previously discussed is used for growth dynamics of phytoplankton blooms. The model is applied to Auke Bay, Alaska, for purposes of example.

The general characteristics of Auke Bay, Alaska, are used in the model to represent the physical environment. However, no attempt has been made to quantitatively simulate a specific series of blooms.

Auke Bay is located in southeastern Alaska about 20 km northwest of the city of Juneau. It is a small embayment, with an area of approximately 11 km², off a system of large fjords which connect the open ocean. Depths of 40 to 60 m predominate in the upper end of the bay.

Water chemistry and biological data were collected at Auke Bay during the spring and summer of 1967 (Bruce, 1969). The water temperature, salinity, and certain nutrient concentrations were measured at the surface, 5, 10, 20, 30, and 50 m depths. These data revealed significant changes occurring in the water column during the spring bloom (April through June). The temperature increased progressively (from 3 to 13 C at the surface) and the salinity decreased (from 30 to 15‰ at the surface) resulting in a strongly stratified water column. During the same period, a succession of diatom blooms occurred. The nitrate, phosphate and silica concentrations in the upper 10 m were reduced markedly during the growth phase of the first bloom and remained at low levels through the summer. Nutrient concentrations below about 20 m were not significantly affected. The relative concentration of the nutrients in the epilimnion indicated that nitrate was limiting. It is assumed for the purposes of this study that horizontal variations in the environment are negligible compared to the vertical gradients and that mechanisms in the Bay can be generally represented by considering a typical water column.

The Model

General

The time-space distribution of materials suspended in the water of a channel with constant cross-sectional area is frequently represented by the following one-dimensional differential equation:

$$\frac{\partial C}{\partial T} = \frac{\partial}{\partial Z} \left(D \frac{\partial C}{\partial Z} \right) - \frac{\partial}{\partial Z} (UC) + S \quad (5)$$

where C = concentration of material, T = time, Z = distance, D = dispersion coefficient, U = advective velocity and S = time rate of addition (or removal) of material. The first term on the right-hand side of Equation 5 is the dispersion term and represents the transport of materials due to mixing. The second term represents advective transport and the third term represents a source (or sink) for the material. An explicit, finite-difference scheme was developed for the solution of Equation 5. Following is a general summary of the model; however, specific techniques can be found in references (Grenney, 1972).

The water column is conceptualized as being divided into segments of equal depth ($\Delta Z = 1.5$ m). Because no significant changes occurred in temperature, salinity, or nutrient concentrations below 30 m, only the top 30 m of the water column were modeled. Each segment is a completely mixed subsystem and reactions within each segment are calculated over a finite-time interval ($\Delta = 2$ hours).

During each Δ the program proceeds through three specific steps. The first is to calculate the growth of each phytoplankton species and the resulting environmental nitrate removal independently in each segment. This step is accomplished by means of a subprogram of the multicompartiment model developed in the previous section. The second step is to advect the various phytoplankton species between segments in accordance with their respective sinking velocities. The third step is to disperse the environmental nitrate and the organisms between segments as a function of the dispersion coefficient at the interface. When suitable values of ΔX and Δ are selected, this stepwise procedure has been shown to be efficient and to provide reasonably accurate solutions.

Growth

Growth as described by the subcompartment model represents organisms growing at optimum levels of temperature and light in a laboratory. In the natural environment, however, variations in temperature and light will cause significant variations in the biological reaction rates.

Light

Light provides the ultimate source of energy for the organisms and, therefore, affects the rates of the intracellular growth processes. There is evidence to indicate that the individual process rates are not affected to the same degree by light variations. Each of these reaction rates could be treated individually in the model if the proper functions were known; however, the

influence of light on the net growth rate is considered to be of sufficient accuracy for the study. This is accomplished by multiplying the net growth rate at optimum light by a dimensionless factor, F_l .

The attenuation of light with depth can be approximated by an exponential function:

$$I = I_s \exp(-kZ) \quad (6)$$

where I is the light intensity at depth Z and I_s is the intensity at the surface. Auke Bay has a relatively shallow photic zone (1% light level) of about 12 meters which is represented in the model by using a value of k equal to 0.38 per meter.

Experimental evidence indicates that the photosynthetic rate increases with light intensity and will reach a constant, maximum rate for a particular range of intensities (Eppeley et al., 1969; Ryther, 1956; Ryther and Yentsch, 1958; Sorokin and Krauss, 1958; Thomas, 1966; Yentsch and Lee, 1966). The photosynthetic rate is inhibited at light intensities above this optimum range. Some mathematical models have proposed linear variation of photosynthesis with light intensity (Steele, 1958; Riley, 1946); some have proposed a hyperbolic function (Chen, 1970; Middlebrooks and Porcella, 1971); and others have proposed an exponential function (DiToro et al., 1970). Relative photosynthetic rates for diatoms were calculated from the average curves reported in these references and are shown in Figure 6(A) where

$$f_l = \frac{P}{P_{\max}} \quad (7)$$

P = photosynthetic rate at light intensity I , and P_{\max} = maximum photosynthetic rate. These data were fit by eye with the equation

$$f_l = 1 - \exp(-0.0224I) \quad (8)$$

as shown by the solid line in Figure 6(A).

Figure 6(A) approximates the instantaneous growth rate, relative to the maximum, at a particular light intensity. During a 24-hour period the light intensity will vary drastically and, since instantaneous light intensities are not represented in the model, it is necessary to estimate an average daily value of f_l from an average daily value of light intensity. The diurnal variation in light intensity on the surface can be approximated by the cosine law (Berry, 1945):

$$I = I \cos\left(\frac{\pi}{T_l}T\right), \quad -\frac{1}{2}T_l \leq T \leq \frac{1}{2}T_l \quad (9)$$

$$I = 0, \quad \text{otherwise} \quad (10)$$

where I = instantaneous light intensity, T = time measured from local noon (days), T_L = length of daylight period (days), L = light flux at noon (langleys per day). Substituting Equations 9 and 10 into Equation 8 gives:

$$f_l = 1 - \exp(-0.0224L \cos \theta), \quad -\frac{\pi}{2} \leq \theta \leq +\frac{\pi}{2} \quad (11)$$

$$f_l = 0, \quad \text{otherwise} \quad (12)$$

$$\theta = \frac{\pi}{T_L} T \quad (13)$$

The time average value of f_l over a day can be represented as

$$F_l = \int_{-T_L/2}^{+T_L/2} \left\{ 1 - \exp \left[-0.0224L \cos \left(\frac{\pi}{T_L} T \right) \right] \right\} dT \quad (14)$$

Equation 14 can be reduced to the form:

$$F_l = T_L - \frac{2T_L}{\pi} \int_0^{\pi/2} \exp(-0.0224L \cos \theta) d\theta \quad (15)$$

The average length of the daylight period at Auke Bay during the study period was estimated to be about 2/3 days (Berry, 1945). The value of L can be estimated from the following equation:

$$L = \frac{L_D \pi}{2T_L} \quad (16)$$

where L_D = total langleys on the surface during the day. The integral in Equation 15 was solved numerically for various values of L_D and the results are shown as the dashed line in Figure 6(A).

The surface insolation (L_D) at Auke Bay during the study period averaged about 175 langleys per day (Bruce, 1969). Values of L_D were calculated at various depths by Equation 6 and then the dashed curve in Figure 6(A) was used to determine the average daily relative growth rate (F_l). The resulting values of F_l [shown in Figure 6(B)] were used in the model to represent the influence of light on the phytoplankton community.

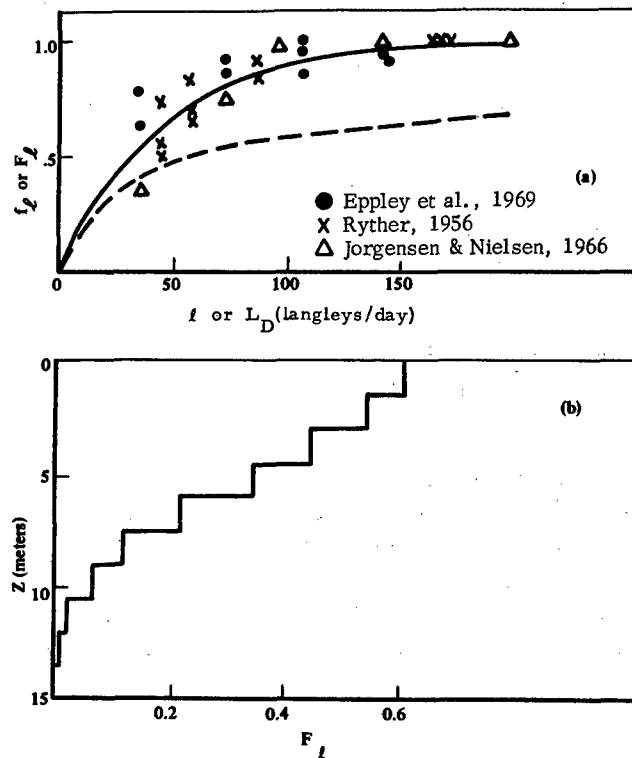


Figure 6. Relative effect of light intensity on growth.

Temperature

The variation in growth rate with temperature has been represented in mathematical models by exponential equations of the following general form:

$$\frac{\mu_1}{\mu_2} = K_\tau (\tau_1 - \tau_2) \quad (17)$$

in which μ_1 and μ_2 = specific growth rates at temperatures τ_1 and τ_2 respectively and K_τ = a constant (Middlebrooks and Procella, 1971; Riley and Von Arx, 1949). The justification for this approach is based on the observation that enzymatic reaction rates tend to increase exponentially with temperature. A plot of saturated growth rate vs. temperature has been constructed from data from a number of sources (DiToro et al., 1970). These data were widely scattered, however, and no functional relationship was apparent.

The variation in growth rate as a function of temperature at constant light intensities has been reported for three species of tropical oceanic phytoplankton (Thomas, 1966), and the diatom, *Nitzschia closterium* (Spencer, 1966). These data were normalized by calculating the following dimensionless variables:

$$F_{\tau} = \frac{\mu}{\mu_{\max}} \quad (18)$$

$$\tau_r = \frac{\tau - \tau_0}{\tau_{\max} - \tau_0} \quad (19)$$

where μ = growth rate occurring at temperature τ , μ_{\max} = maximum possible growth rate, τ_{\max} = the temperature at which μ_{\max} first occurs, and τ_0 = temperature at which zero growth occurs. In some cases τ_0 and τ_{\max} had to be extrapolated from the data. F_{τ} = the relative growth rate and τ_r = the fraction of the temperature range between τ_0 and τ_{\max} . Values of F_{τ} vs. τ_r are shown in Figure 7. These data were fit by the following second order polynomial,

$$F_{\tau} = 1.92\tau_r - 0.92\tau_r^2, \quad 0 \leq \tau \leq \tau_{\max} \quad (20)$$

as shown by the solid curve in Figure 7.

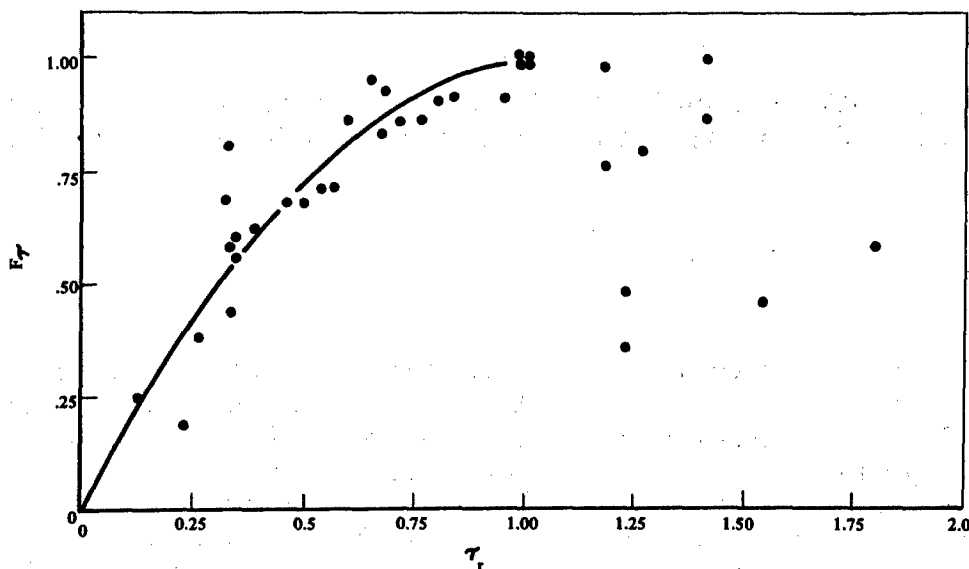


Figure 7. Relative effect of temperature on growth.

The optimum temperature for cold water diatoms is probably in the neighborhood of 13-16 C (Hutchinson, 1967; Jorgensen and Nielsen, 1966) so values for τ_0 and τ_{\max} were assumed to be 0 and 13 C respectively for the Auke Bay model. Average weekly temperatures were estimated from the field data for each segment in the water column at Auke Bay. These values were stored in a matrix in the computer memory for use in Equation 20 during each model run. The maximum temperature occurring in Auke Bay during the study period was 13 C, so the range of Equation 19 is sufficiently large.

Convection and Dispersion

If the organisms have a negative buoyancy, they will have a downward velocity relative to the water. This sinking velocity is designated by the symbol U in Equation 5 and represented the advection of organisms between segments in the model. Phytoplankton sinking velocities are related to the physiological state of the organism and to water density (Lung, 1959; Munk and Riley, 1952; Riley, 1965). However, at this time a functional relationship between cell storage and sinking velocities has not been defined and, therefore, U will be considered constant over the study period.

Methods have been developed to estimate the dispersion coefficients (D) at various depths in the water column from changes in the temperature or salinity profiles (Bella and Grenney, 1972; Orlob and Selna, 1970). These methods are based on the assumption that all of the vertical transport of heat or salinity in the water column can be represented by a diffusive-type expression. Consider any cross section in the water column at depth Z . Diffusive transport can be expressed by:

$$- (D \frac{\partial \theta}{\partial Z})_Z = R_Z \quad (21)$$

where Z is the depth, θ is the temperature (salinity), and R_Z is the rate change in total heat (salt mass) below depth Z . As an approximation one may write:

$$- (\bar{D})_Z \frac{[\theta_{(Z+\Delta Z/2)} - \theta_{(Z-\Delta Z/2)}]}{\Delta Z} = \frac{H(t) - H(t+\Delta t)}{\Delta t} \quad (22)$$

where Δt is a finite time interval, $H(t)$ is the total heat (salt mass) below depth Z at the beginning of time interval Δt , and $H(t+\Delta t)$ is the total heat below depth Z at the end of the time interval. The bars denote values averaged over the time interval. It is assumed that flux in or out of the water column can occur only at the upper boundary, i.e., the top segment.

Dispersion coefficients were calculated at each water column segment from average weekly temperature and salinity data for Auke Bay. Changes in the salinity profile did not occur in sufficient magnitude to calculate D until the first of May. Values of D calculated from the temperature profile from May to July were generally one and a half to three times larger than corresponding values calculated from the salinity profile. This relationship might be expected because no attempt was made to account for changes in the temperature profile due to the penetration of radiant energy. Also, error is undoubtedly introduced by the assumption in the estimating procedure that no horizontal flux into or out of the water column occurs except in the top segment. Values of D estimated from the salinity profile were used in the model for the period May to July. Values of D estimated from the temperature profile were used for the month of April. These values were adjusted down by factors of three-fourths to one-half in order to be consistent with the values calculated from the salinity data. The average weekly dispersion coefficients for each segment were stored in an array in the computer memory for use during the model runs.

Profiles were calculated from the estimated dispersion coefficients for the given boundary conditions in order to check the estimating procedure. Comparisons were made between the profile used to estimate the coefficients and the profile generated by the estimated coefficients. The salinity profiles agreed within 3% and the temperature profiles generally within 20%. The dispersion coefficients occurring at mid-April, mid-May and mid-June are shown in Figure 8.

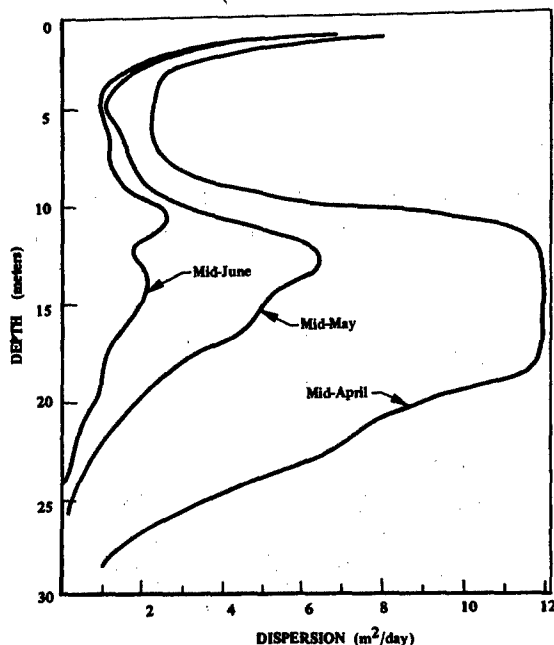


Figure 8. Vertical distribution of calculated dispersion coefficients in Auke Bay.

The curves show the same general shape as some estimated by similar methods for lakes and reservoirs (Bella, 1970; Orlob and Selna, 1970). In general, the dispersion coefficients estimated for Auke Bay are about four to five times larger than estimated for Lake Sammamish, Washington (Bella, 1970). The increasing stability of the water column through the spring bloom is indicated by the decreasing dispersion coefficients in Figure 8.

Model Responses

Several runs were conducted in order to observe the time and space response of the model. The top 30 meters of a water column were modeled using a finite time interval Δ of two hours and segment depths ΔZ of one and one-half meters. The average weekly dispersion coefficients and temperatures estimated from Auke Bay data for each segment were stored in arrays in computer memory. A uniform daily surface insolation of 175 langleys and a 12 m photic zone were assumed to approximate Auke Bay conditions during the study period. The phytoplankton population was described by the multicompartment model developed in the previous section. Also the influence of light and temperature on growth are represented by the methods discussed previously.

The model indicated that phytoplankton populations decrease rapidly with increasing sinking velocity. For example, an increase in U from 0.33 to 1.0 meters per day results in an almost 67 percent decrease in the peak concentration. In all cases, the environmental nitrate concentrations were reduced to very low levels in the upper waters of the photic zone. Low sinking velocities resulted in significantly earlier blooms than high sinking velocities. A phytoplankton population cannot increase when the organisms are sinking out of the photic zone at an average rate equal to or greater than the growth rate. This condition apparently exists during the month of April for the case when $U = 1.0$ m/day. However, as the water warms the growth rate increases and apparently overtakes the sinking rate during the first week in May. When nutrients are depleted in the upper waters the population decreases rapidly.

Early in the season, the growth rates of the populations are limited by the cold temperatures. The model indicates that during these conditions the phytoplankton have a tendency to store large amounts of nutrient as N_1 and N_2 . These intracellular nutrient pools are utilized for growth as the water warms, and when they are depleted the growth rate declines. Intracellular storage reached 75% of total cellular nitrogen when the growth rate is limited by temperature in nutrient rich water. As the season progresses, the water warms and the environmental nitrate decreases causing reduction in storage.

The combination of temperature, light, and sinking velocities may have a significant influence on the vertical distribution of the population especially during periods of low nutrient supply. As the season progresses,

nutrients are depleted in the upper segments of the water column and the growth rate falls below the sinking rate resulting in a decrease in the local population concentration. However, warming tends to increase the growth rate at the depths where intracellular nitrogen is available for growth and, thus, the maximum population has a tendency to occur at greater depths as the season progresses.

The Auke Bay spring blooms and nitrate concentrations for the spring of 1967 (Bruce, 1969) are shown in Figure 9. The nitrate was depleted in the surface water during the first bloom and remained at very low levels for the remainder of the spring. The nitrate concentrations at 10 m (near the bottom of the photic zone) decreased gradually indicating that nitrate at this depth provided a nutrient source during the entire sequence of blooms. Nitrate concentrations were not significantly changed at 20 m.

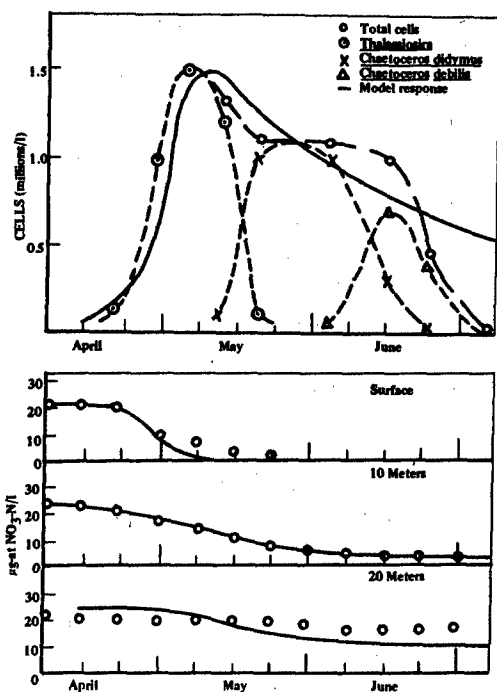


Figure 9. Auke Bay blooms, spring 1967.

The solid line in Figure 9 is the model response for the total cell concentration in a two species system. Comparison of the model response with the field-measured total cell count indicates reasonable agreement during the April-May bloom.

The nitrate concentrations at the surface and 10 m are very similar between the model and the observed values. At 20 m depth, the model shows significantly more change than the field data, a condition which might also be due to exaggerated biological activity in the model at low temperatures.

A closer correspondence between the model and the data could probably be obtained if the following information were known about the phytoplankton species in Auke Bay:

1. Better estimates of the parameters representing storage and growth characteristics of the specific phytoplankton in Auke Bay.
2. More knowledge about sinking velocities and their relationship to intracellular storage.
3. The inclusion of grazing in the model. Significant numbers of zooplankton were observed at the time of population decrease. The fact that grazers are not represented in the model may account for the discrepancy between field observations and model response toward the end of June.

CONCLUSIONS

Ecosystems are systems of living organisms in a physical chemical environment which have developed and adapted over long periods of time. As such, they present unique problems for quantitative description and prediction. Mathematical models can be powerful tools for analyzing these systems. Models are constructed for particular purposes and are subject to numerous limitations. Although it can be concluded that all models are less than perfect, their evaluation should only be based on characteristics in relation to their proposed use. In this regard an optimal balance between accuracy and practicability is sought, and, in fact, is often approached.

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LIST OF SYMBOLS

Compartment Model

BOD	Biochemical oxygen demand
SL	Loading rate of BOD
K _L	Decay rate of BOD
K _R	Rate of bottom deposit or adsorption of BOD
K ₁	Ammonia decay rate to nitrite
K ₂	Rate of conversion of nitrite to nitrate
S ₁	Ammonia loading rate
S ₂	Nitrite loading rate
S ₃	Nitrate loading rate
S ₄	Nitrogen fixation rate
K ₃	Ammonia assimilation rate
K ₄	Nitrite assimilation rate
K ₅	Nitrate assimilation rate
K ₁₁	Yield coefficient to convert mg nitrogen to mg biomass
K ₁₂	Algal settling rate
K _d	Algal death rate
K ₁₃	Rate of BOD return from algal decay
K _A	Surface reaeration rate
K _a	Rate of oxygen addition by photosynthesis
K ₆ , K ₇	Rate of oxygen removal by nitrification
K ₈	Rate of oxygen removal by algal respiration
K ₁₀	Rate oxygen diffusion to sediments
SC	Addition of oxygen by benthic algae
K _O	Release of ammonia to water by decaying algae (dimensionless)
Q	Dilution rate
C _i	Concentration in the influent stream

Subcompartment Model

N ₁	Average concentration of inorganic nitrogen in the cell
N ₂	Average organic nitrogenous intermediates in the cell
N ₃	Average protein in the cell
N _I	Environmental concentration of nitrate
N _O	Excreted nitrogenous organics
G _I	Reaction rates among subcompartments
G ₁	Reaction rates among subcompartments
G ₂	Reaction rates among subcompartments
K ₃	Reaction rates among subcompartments
K ₄	Reaction rates among subcompartments
K ₅	Reaction rates among subcompartments
K ₆	Reaction rates among subcompartments

Component Modeling

f	Flux
N	Potential
t	Time
C	Capacitance
R	Resistance
P	Potential source component - potential energy for growth
N_I	Potential associated with the source
C_3	Capacitance component - storage of biological energy in the living organism
N_3	Potential associated with the C_3 capacitance component
R_2	Resistance component - dissipation of biological energy
N_R	Potential associated with R_2 resistance component
JF	Flux junction
β_1	First order coefficient
\hat{N}_I, \hat{N}_3	Initial conditions

Model Application

C	Concentration of material
T	Time
Z	Distance
D	Dispersion coefficient
U	Advective velocity
S	Time rate of addition of material
I	Light intensity at depth Z
I_s	Light intensity at surface
P	Photosynthesis rate at light intensity I
P_{max}	Maximum photosynthetic rate
T_l	Length of daylight period (days)
L	Light flux at noon (langley per day)
L_D	Total langley on the surface during the day
μ_1	Specific growth rate at τ_1
μ_2	Specific growth rate at τ_2
K_τ	Temperature constant
μ	Growth rate at temperature τ
μ_{max}	Maximum growth rate
τ_{max}	Temperature at which μ_{max} first occurs
τ_0	Temperature at which zero growth occurs
F_τ	Relative growth rate
τ_r	Fraction of the temperature range between τ_0 and τ_{max}

THE ROLE OF BIOASSAYS IN ENVIRONMENTAL RESEARCH

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Through the second quarter of the twentieth century water pollution control efforts in the United States were directed largely toward the abatement of the discharge of raw domestic sewage into the nation's surface waters, primarily as a protective measure against waterborne infectious disease of human beings. The industrial complex of the United States was not nearly as large as it is today, and during that period of time, before the rapid growth of the synthetic organic chemicals industry, the industrial wastes that were discharged were substantially more simple in makeup. Most of the problems associated with domestic sewage were oxygen depression, ammonia, and sludge beds, rather than the highly toxic chemicals that are present in many of today's waters. Water pollution biologists were involved primarily in documenting damage to organisms so that these gross situations could be corrected.

During the present quarter century efforts have been shifted from correcting already existing bad situations to preventing wastes from being discharged. This shift in emphasis has brought about the need for predictive tools so that the degree of treatment and the permissible amount of waste materials dischargeable to receiving waters can be determined when the plant is under construction. Furthermore, waste-treatment research efforts are being directed toward removing those materials that are most harmful to organisms, including man. We therefore need to know acceptable levels of many different kinds of pollutants. If the treatment facilities are going to be constructed at the same time the plant is being built, field studies are not useful for establishing the degree of treatment needed. This is one role bioassays play in today's water pollution control efforts.

One must realize that today a single plant manufacturing synthetic organic chemicals discharges literally dozens, if not hundreds, of chemicals that are formed during the synthesis process or formed when the wastes are combined before discharge. It is almost certain that many of these chemicals have not yet been identified, and very little is known about the variability and the ratios of one to another during the operations. Even if the exact makeup of these wastes and the variations in the ratio were known, still almost no information would be available to assess the significance of these waters to organisms, because very few data at hand pertain to mixtures of chemicals, and particularly mixtures that vary from time to time.

The obvious answer would appear to be complete chemical analyses to determine waste composition. Such analyses are becoming more feasible as advanced analytical techniques such as the mass spectrometer-GC-computer systems are being developed. We may ignore the immense cost and extreme degree of expertise needed to interpret the results of such instruments, but we must recognize that even these tools are not all-inclusive and cannot tell us much about the biological effects of the combined chemicals present in a wastewater.

Another important aspect of interpreting the effect of chemicals in surface waters on organisms is the many forms in which a given chemical may occur. Waste-treatment engineers and water pollution biologists too often regard chemicals as being present in a single form and do not consider that most materials present in water will occur in various forms, including ionic, molecular, precipitated, sorbed, or complexed with organic matter. Each of these forms must be considered as a separate toxicant. Even elements such as metals occur in many different forms and oxidation states, and the effects of one form may be entirely different from those of another. Such problems often are ignored in bioassays, and the validity of the conclusion drawn may be brought into question.

Toxicologists who have worked primarily with warm-blooded vertebrates may not appreciate the immense range of sensitivity that occurs among aquatic organisms. It is common to find a difference in sensitivity among organisms to a given pollutant on the order of 100 to 1,000 times. Even larger differences have been found among some of the synthetic organic chemicals, particularly the organophosphate insecticides. The mammalian toxicologist does face the problems of extrapolating results from experimental animals to humans, and obviously the margin for error is smaller in these extrapolations than it would be for the protection of aquatic organisms. However, it is not individuals or single species that are being protected in the aquatic environment, but rather communities

that involve the interrelationships of dozens or hundreds of different species of organisms in any one given locality. The aquatic toxicologist, therefore, is not only faced with extrapolating results from experimental animals used in the laboratory to other species present in natural waters, but is further faced with the problem of extrapolating from individuals or small numbers of individuals to populations of many different species interacting with each other. The prediction of effects in the field from controlled laboratory conditions is very difficult.

In the above comments I have used many times the words organism or biological effect, and some of you may wonder at this emphasis. A moment's reflection will reveal that almost all reasons for controlling water pollution are biological. Examples are: swimming, boating, drinking water supplies, fishing (both sport and commercial), and stock water supplies. Only industrial usage of water does not often involve directly biological organisms of some type. For this reason chemical or physical analytical procedures are only tools used to measure the degree of pollution, and they will have little meaning if they are not related to the response of organisms. This often comes as a shock to chemists who are asked to relate the significance of their chemical measurements to the biological response expected from the concentrations measured.

We should view the aquatic bioassay as another analytical tool that can help us describe the significance of various pollutants in the water at a range of concentrations. The data available to us at present are meager, but it is clear that the variation in chemical constituents in wastewaters as determined by the usual chemical methods often bears no resemblance to the toxicity of that same waste as measured by some biological response. There is urgent need, therefore, for today's water chemists to make their analytical techniques more biologically meaningful.

Toxicology work with the complex wastes that are a large part of the wastewater problem of today's industrial society must be substantially different in approach and viewpoint from classical toxicology work of the past. It is a very difficult and challenging task to do meaningful toxicology work with a "toxicant" (an industrial wastewater) that is of unknown composition and likely to be different each time a new sample is obtained.

Consider for a moment some of the ramifications of working with such a waste. On any given day materials present in the waste may have a high short-term, or acute toxicity, and a low long-term, or chronic toxicity. For such wastes perhaps short-term, or acute tests are most meaningful.

On the next day, or some day following, the wastes may be composed primarily of materials with low acute toxicity and high chronic toxicity. Perhaps only long-term, or chronic tests will fairly assess the significance of the toxicity of this waste. On some days or in some wastes chemicals may be present that are most important in terms of man's use of the water because they produce unacceptable residues in the organisms living in the water, but they produce apparently no toxicity to the organisms sorbing them. Polychlorinated biphenyls and some of the chlorinated pesticides are examples of such chemicals. In studies completed at the National Water Quality Laboratory, for example, we have found that water concentrations of methylmercury below 100 nanograms per liter appear to produce no adverse effects in a variety of species, even during full life cycle exposure studies. On the other hand, we know that concentrations as low as 15 nanograms per liter produce residues of methylmercury in the tissue that exceed the half-part per million action level established by the Food and Drug Administration.

In addition to the standard toxicity tests mentioned above, the aquatic toxicologist assessing the impact of a mixed industrial waste must also consider the attraction or avoidance that may be displayed by the organisms in the receiving water. It matters little whether or not an organism can thrive in a particular concentration. If it will not stay in that concentration and in that area, that portion of the receiving water is useless as living space. The importance of avoidance has only recently been appreciated by many of us. In a study recently completed at our Newtown Fish Toxicology Station near Cincinnati, Ohio, we found that avoidance was the most important effect of the chemical on the organisms in the stream. We introduced copper sulfate at a concentration of 100 micrograms per liter into a natural stream in southwestern Ohio near Cincinnati. Laboratory studies showed that this concentration was sufficiently high to inhibit reproduction in the more sensitive species, but would permit reproduction in the more resistant ones. We found that our predictions were good but we had not measured, in the laboratory, the avoidance response of fish toward copper. As soon as copper was introduced into the stream, the exodus of fish, particularly the sensitive ones, from the test portion of the stream was so great that the populations of these species were decimated. The animals were not sick or weakened from the exposure, they were simply leaving. Congregations of fish were observed in areas where spring water entered the stream and where the concentrations of copper were measurably lower than in the other part of the test portion. The white sucker was among the species more sensitive to copper in this study, and the only two recorded spawnings of white suckers in the test portion of the stream occurred in the most downstream end of the test portion where the concentration of copper was lower than at the head portion.

Although white suckers are riffle spawners, these fish spawned in pool conditions, one at each end of the dam that blocked their further downstream movement. Perhaps this particular choice of spawning site was coincidental, but non-scientists would say that they "went as far as they could to spawn."

Another difficult problem that must be addressed when extrapolating results of aquatic bioassays to natural communities is the interaction of the species with each other, especially the food-chain organisms. Many studies have been completed on the food habits of various important species that are protected by present water pollution control laws. It is difficult to find food-chain organisms that are essential to the well-being of higher trophic levels. Most of our desirable fish species appear to be opportunists and feed, within limits, on what is available. Therefore, it is difficult to find representative food-chain organisms for use in aquatic bioassays. Furthermore, the food-chain organism group is the most diverse assemblage of organisms, and there is no good toxicologic reason to expect the response of an aquatic insect to be comparable to that of a diatom, protozoan, or rotifer. The aquatic toxicologist is continually faced with the problem of selecting the right organisms to test from a much larger array of organisms, a bigger problem than for the mammalian toxicologist concerned with only human health effects.

A third problem faced by the aquatic toxicologist is the relating of the toxic effects of a given chemical or waste to variable receiving water characteristics. It is well established in the literature and has long been known that the alkalinity, in hardness, and pH of a water may drastically affect the toxicity of heavy metals, such as lead, to aquatic organisms. The effect of alkalinity on lead toxicity may approach a factor of 100 from one type of water to another. Such ranges in toxicity must be considered when one is trying to predict effects in a natural receiving water. In recent years substantial progress has been made in developing selected ion electrodes and other analytical techniques that can measure the species of heavy metal in the water, rather than giving a total metal determination. Some of these techniques look very promising and make it seem more feasible now than ever before that the measurement of these materials can be made in such a way that they will relate to the biological effect. Better measurements may eliminate much of the apparent variable toxicity that is observed, particularly in regard to metals in various types of water.

In addition to the problem of variable receiving water characteristics, we have also the problem of variable flow in the receiving water, and consequently the concentrations of pollutants actually occurring in the stream or lake are never the same. Constant-level-exposure bioassays are therefore somewhat less realistic and more difficult to relate to natural conditions. The whole area of intermittent or variable exposure levels in aquatic toxicology has really not been tackled as yet. The statistical designs required for such bioassays are very difficult to develop in such a way that the results will be meaningful, and at the present time, only a few laboratories are beginning work in this direction. What little work has been done with such variable exposures has used such excessively high concentrations to shorten the test and thereby complete more tests that the results obtained are essentially meaningless for use in regulatory activity. What we need are tests of intermittent exposures that use concentrations approaching the "no-effect" level. It is here that we are interested in controlling toxic effects, and reactions produced by such low levels over longer periods of time are likely to bear little resemblance to those produced by high level acute exposures that result in death in a matter of minutes or hours.

The techniques for conducting aquatic bioassays are probably as advanced and sophisticated as bioassays using mammals and birds. I suppose that the chief unanswered questions that face the aquatic toxicologist are not greatly different from those of the mammalian toxicologist of today, namely, extrapolation of results from one organism to another. I would only submit that the additional complication faced by the aquatic toxicologist is extrapolation of results from experimental animals to other communities where the interaction among species is an added new dimension not normally encountered in human toxicology.

With the issuance of permits for discharge of wastes to surface waters brought about by the 1972 Water Quality Act, the need for aquatic bioassays on industrial effluents is becoming more and more apparent to many people. The conduct of the bioassay is no different whether the toxicant under study is a pure chemical or a complex industrial waste. What is different is the extrapolation of these results in a meaningful way to predict the effects that are going to occur in the natural water to which the waste will be discharged.

ORGANOPHOSPHATE PESTICIDES:
THE RESIDUE INTOXICATION PROBLEM

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Shortly after the introduction of the organophosphate pesticides in the U.S., it became apparent that it was possible to become clinically intoxicated from exposure to the residues of these compounds on soil or foliage days or weeks following application. From the earliest days of O.P. usage, California seemed to be the center of reported incidents of residue intoxication, with a large proportion of incidents involving parathion on citrus crops marking this combination as an area of major concern.

The increasing awareness of the existence of the residue hazard to the large agricultural workforce in California caused the Director of the State Department of Agriculture to assemble an advisory committee of occupational health and agricultural specialists who, in 1970, recommended the establishment of "field worker re-entry intervals" as a protective measure. These intervals, which define the passage of time between pesticide application and entry into the field for activity involving substantial foliar contact, came into force as State regulations in June of 1971 (California State Department of Food and Agriculture, 1971). These regulations seem to have reduced the incidence of further outbreaks of residue intoxication.

In February 1972, a Task Force was constituted under the auspices of the Federal Working Group on Pest Management to survey the extent of occupational hazards due to pesticides and, in particular, to consider the residue intoxication problem from a national perspective. The report of this Task Force will emerge shortly but its formation was only the first of several major moves by the Federal Government signaling increased concern with the residue intoxication hazard. In recent months, the Occupational Safety and Health Administration and the Environmental Protection Agency have separately and jointly announced an intention to promulgate national standards involving re-entry intervals and have held hearings regarding such standards. All of these hearings have cited the lack of relevant technical data, particularly data concerning the extent of apparent regional variations of the hazard. Of equal concern is the lack of concise human exposure data relating what little available foliar residue data exist to either absorbed doses or physiological responses.

In February 1972, the National Institute for Occupational Safety and Health awarded a research grant to our group at the University of California in Berkeley to investigate the technical questions surrounding the residue intoxication phenomenon including the development of relevant sampling techniques and experimental procedures needed to evaluate harvester exposure. We are now in the midst of the third year of our project, and we feel that we have come to understand some of the factors influencing the occurrence and severity of this occupational health hazard.

Prior to reviewing some of the results of our work, it is useful to describe our perception of the residue intoxication problem. The diagram of Figure 1 summarizes our view of the phenomenon and provides a frame of reference for the discussion (Popendorf and Spear, 1974).

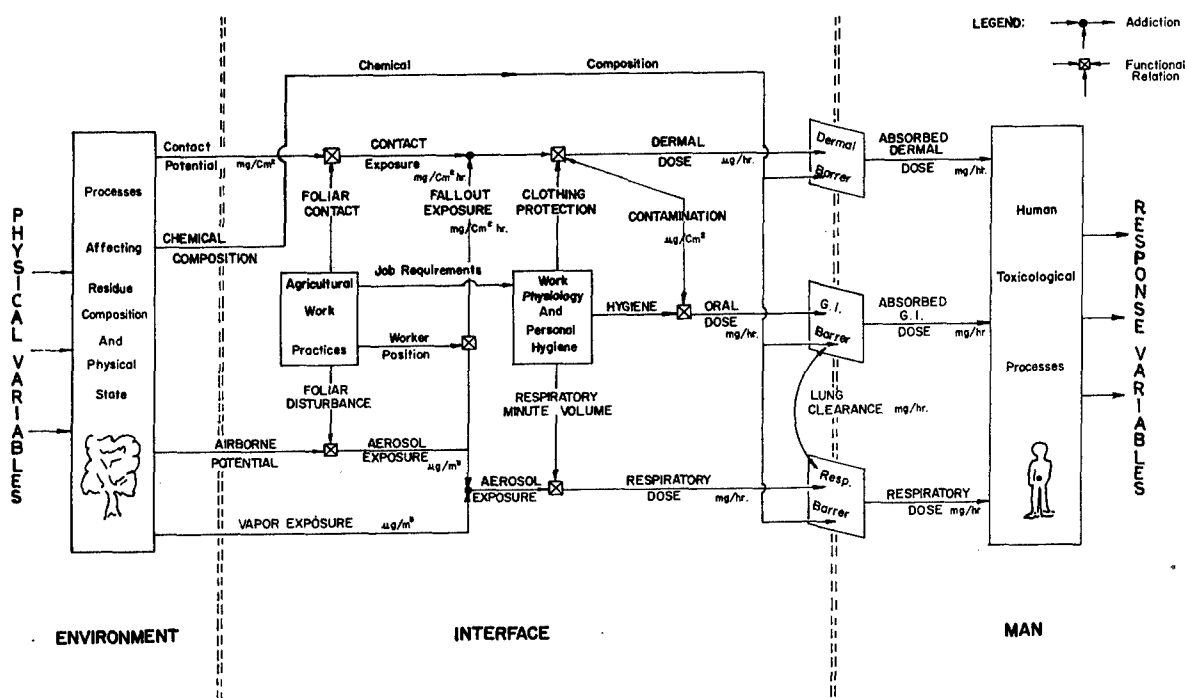


Figure 1. Factors affecting residue intoxication.

On the one side of the diagram is indicated the workplace to which the pesticide is applied. Various physical variables such as the nature of the crop, foliar dust, the specific pesticide and the climate will, over time, interact to alter both the chemical composition of the material and also its physical availability to be transferred to the worker by his activities. The physical nature of the residue will determine its availability and thereby the route or routes of worker exposure. The chemical composition will influence the penetration rates of the pesticide through the body barriers and its subsequent toxicity. At this time there is very sparse information on the effect of most environmental variables on foliar residue decay rates. This is of great importance with regard to the possibility of applying the results of field experiments obtained in one part of the country to another where the environmental conditions may be dissimilar.

Central to the diagram are two blocks representing the influences of the specific job and of various human factors. For example, the first category would involve a description of contact with the foliage that may be specific to the activity; e.g., picking oranges presents a different exposure to residues than does thinning peaches. A variety of personal practices which are also important to residue exposure are easily specified. For example, the access to and use of handwashing facilities prior to eating, the maintenance of reasonably clean clothing and the individual's smoking habits may all play a role. Of equal importance in attempting to estimate dose potentials are variables concerned with work rate. For example, sweat rate may be an important variable associated with dermal absorption of the residues. Clearly, there can be great personal variations in these variables even for groups doing the same work in the same climatological environment.

On the right side of the diagram is indicated the deposition of the residue onto the primary body barriers, its absorption into the bloodstream and the various physiological and biochemical sequelae. This portion of the scheme conceptually encompasses the vast amount of data on the toxicology of the compounds as well as that associated with the dermal, respiratory and gastrointestinal absorption parameters. There are several potential response variables which can be monitored, blood cholinesterase being the most common. In addition, the urinary metabolites of the O.P. compounds present a promising means of quantifying absorbed dose which, when measured simultaneously with enzyme inhibition, should lead to important new insights into the toxicological processes involved.

To date we have conducted seven field studies of various aspects of this problem, two of which involved the exposure of volunteers in controlled experimental situations. Most of our work, including both exposure studies, has been conducted in orange groves treated with parathion. Our first study, however, concerned the measurement of residues of two O.P. pesticides on grape foliage and led to a discovery which appears to be central to understanding the problem (Leffingwell et al., 1974). Figure 2 shows one set of results

from the grape study which indicate the presence of appreciable quantities of the oxygen analog of the pesticide Zolone. Insofar as experiments with rats show the dermal toxicity of Zoloxon to be about ten times that of the parent compound, these findings led us to take a closer look at the oxons in subsequent work.

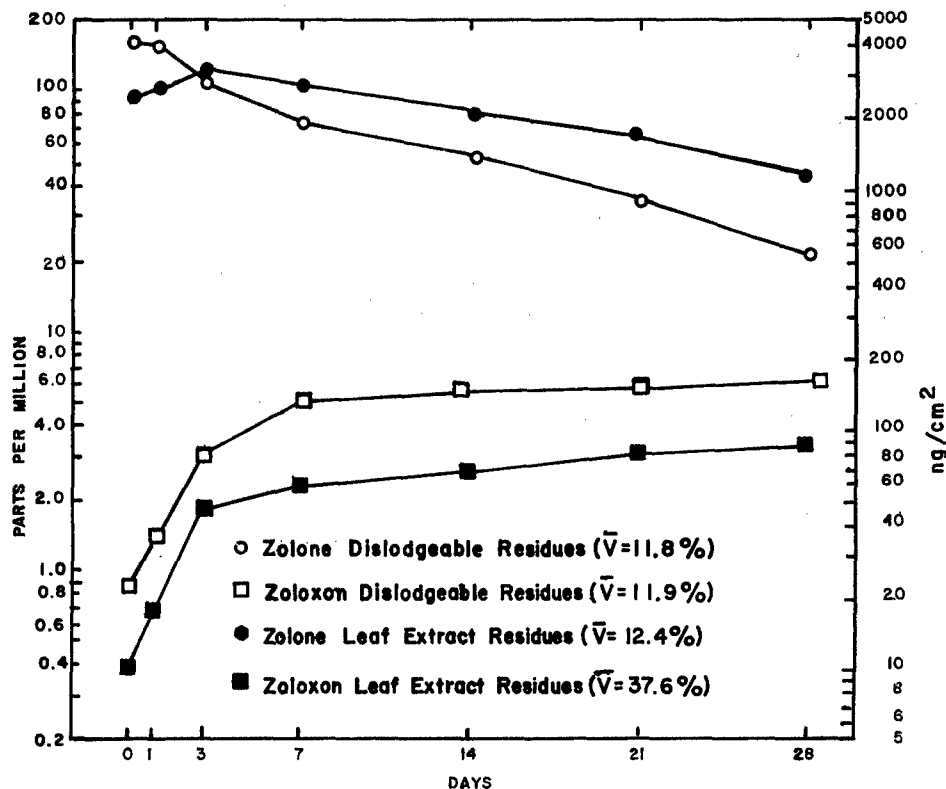


Figure 2. Zolone dilute residues.

A second environmental factor which appeared important to other investigators and to us from our earliest field investigations concerned the high levels of foliar dust which can become aerosolized by the worker during harvesting activities. Using personal air samplers on professional pickers, we measured dust levels on the order of 10 mg/m³ in grapes, 30 mg/m³ in peaches and 40 mg/m³ in orange groves (Pendorff and Spear, 1974). These findings led us to develop a method for collecting samples of this foliar dust with the accompanying pesticide that would be correlated with the airborne exposure potentially available to the workers, a report of which is presently in review. This procedure was used extensively during the summers of 1973 and 1974 in citrus groves. The overall results of the

1973 work are shown in Figure 3 which clearly indicates the dust building over the summer on citrus foliage in Tulare County, California. It is interesting to note that the zero dust intercept of the regression line occurs on April 4. The last significant rainfall occurred on March 26 with minor showers on April 30.

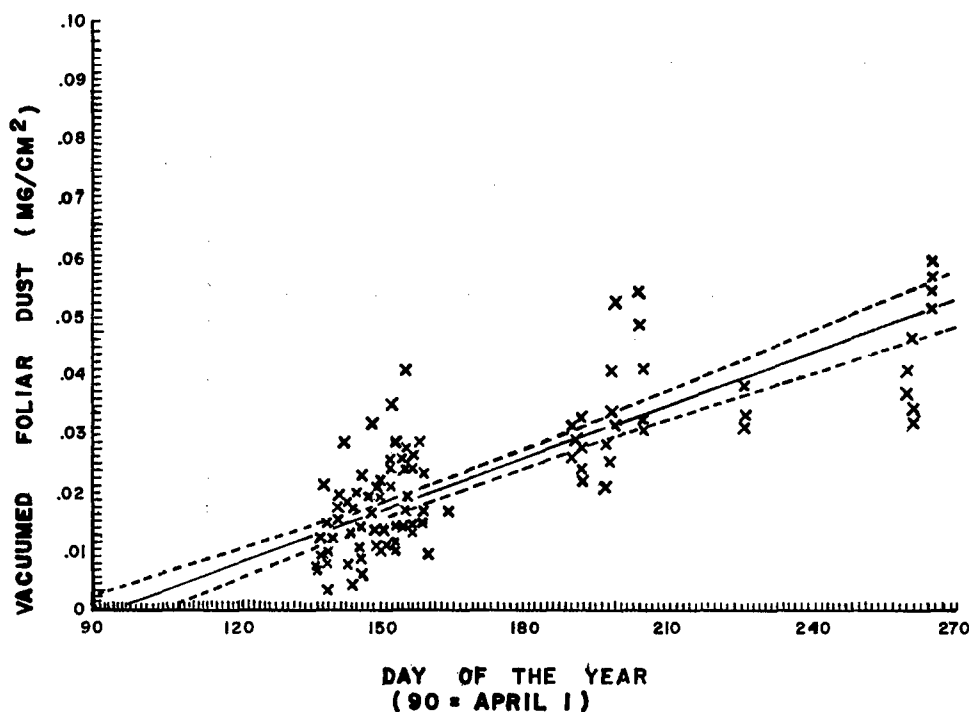


Figure 3.

Keeping in mind the potential importance of the oxons and the possible role of foliar dust as a vehicle to carry them to the worker, we planned a human exposure study, one of the goals of which was to determine whether the dermal or the respiratory route was the principal avenue of worker exposure. This field study, which took place near Orange Cove, California, in July of 1973, focused on the exposure of a crew of six workers harvesting oranges in parathion-treated groves. Entry was made by this crew into three different plots at 21, 24, and 28 days post-application. Among the variables recorded were the foliar residue samples, heat stress indices, work rates and airborne pesticide levels using personal air samplers. Fifteen blood samples were taken from each exposed individual during the three weeks of the experiment. Cholinesterase assays were by the Voss and Sachsse adaptation of the Ellman method (1970).

At that time there was virtually no information on the persistence of parathion on citrus foliage, let alone on that of paraoxon. Figure 4 summarizes one set of our residue results using the dislodgeable residue technique of Gunther et al. (1972). Two features of these data are of interest, the remarkable paraoxon levels and their lack of substantial change over the three exposure periods. The cholinesterase response, shown in Table 1, reflected that the three exposures were quite similar in degree.

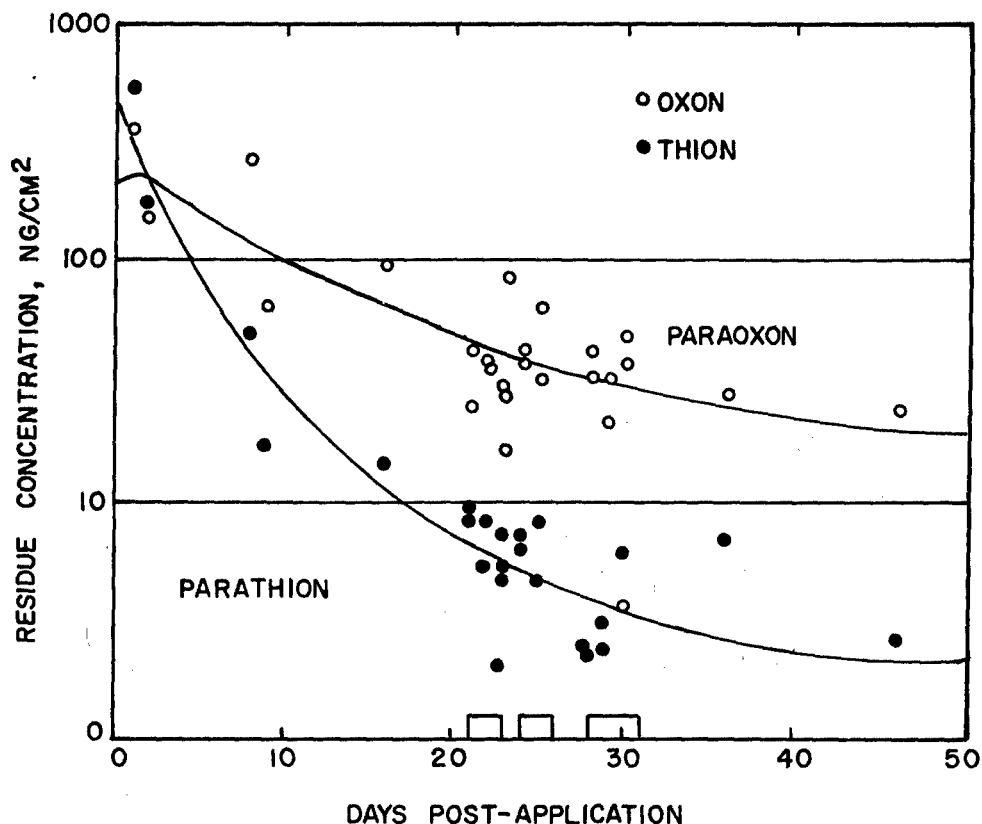


Figure 4. Summary of dislodgeable residues, Tulare II, July 1973.

In an attempt to determine the importance of the respiratory versus the dermal route of exposure, we experimented with various types of half mask respirators with the object of excluding the respiratory exposure. We had no idea how successful we would be in keeping the masks on the men while picking, so we started with disposable paper types and progressed to the more uncomfortable rubber half-mask types with canisters as the experiment proceeded. The canister types were used by half the men for the entire exposure sequences in weeks 2 and 3. At no time could any difference be seen in cholinesterase response between those with and those without respirators.

TABLE 1. EXPOSED GROUP RESPONSE, TULARE II, JULY 1973

Reentry Interval (DPA)	Hours Worked	Exposed Group AChE RBC	Mean Dislodgeable Residues	
			Parathion ng/cm ²	Paraoxon ng/cm ²
21-22	4, 4	- 8.1%	7.35	34.4
24-25	4, 4	-15.9%	6.23	40.3
28-30	4, 4, 6	-10.3%	3.02	33.8

A second result of interest from this study which bears on the dermal dose was obtained using patches affixed to the clothing and the skin. An extra shirt of the type provided the crew was cut into patches and affixed externally to the upper arm area of each man's shirt. Gauze pads backed with aluminum foil were affixed to the skin under the shirt and several inches lower on the arm than the external patches.

Table 2 shows the data resulting from compositing all six samples on each of five days. These data suggest the preferential transmission of paraoxon through the shirts, a finding which illustrates the importance of the oxons.

TABLE 2. COMPOSITE SHIRT AND GAUZE PATCHES, DERMAL DOSE ESTIMATE, TULARE II - JULY 1973

DPA*	Parathion, ng/cm ²			Paraoxon, ng/cm ²		
	Inner + Outer	Inner Only	Percent Penetration	Inner + Outer	Inner Only	Percent Penetration
24	9.25	1.1	12%	30.7	22.4	73%
28	8.85	1.9	21%	38.9	19.6	50%
29	17.2	5.7	33%	47.4	25.9	55%
30**	26.3	6.5	25%	121.6	58.7	49%

Mean Penetration: 23%

Mean Penetration: 45%

* Days Post-Application

** Six Hours Exposure

Overall, this experiment yielded a considerable amount of data, the analysis of which is only now approaching completion. Attempts were made to correlate the various environmental measures of hazard with crew response with limited success due to the essential uniformity of the three fields. However, one correlation proved of considerable interest. Figure 5 shows the mean amount of pesticide collected during each exposure sequence on the membrane filter personal air samplers (which were worn twice a day by each man) plotted against the mean RBC cholinesterase decline per hour of exposure. We feel that these data suggest that airborne dust is the vehicle which conveys the pesticide to the worker and that paraoxon is the principal toxic constituent of the residue. This result, coupled with the respirator results, suggests that the exposure in this situation is principally via the dermal route. In the context of Figure 1, this is termed "fallout" exposure and our current hypothesis is that it constitutes the principal exposure mechanism in California citrus groves.

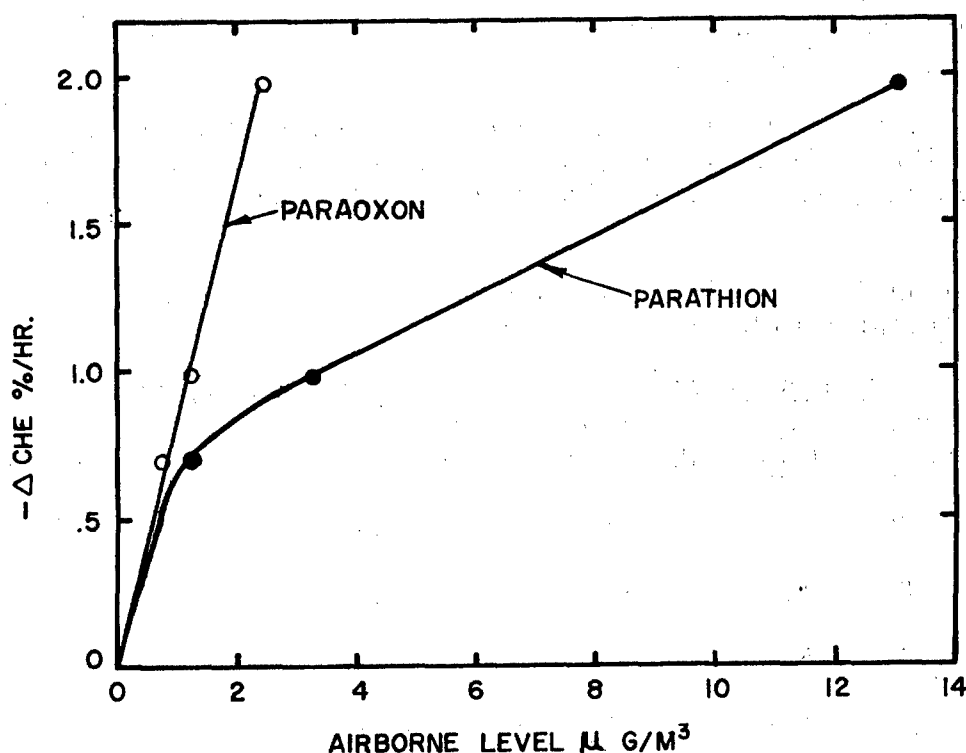


Figure 5. Tulare II mean weekly Δche vs mean weekly airborne level.

Among the problems encountered in our first experiment were the small number of workers in the crew, the variance in the cholinesterase analysis and developmental nature of many of our environmental sampling procedures. In

the past year we have resolved many of these problems and applied both re-fined techniques and our substantially improved knowledge of the behavior of parathion residues of citrus foliage to the design and execution of a second exposure study.

The second study was carried out near Lindcove, California, in July of this year. We increased the crew to nine men, used four unexposed controls for the blood and urine work and set up the plots to give a much wider variation in foliar residues. The laboratory analysis of the environmental samples collected at Lindcove is only beginning, but the first results suggest that our previous hypotheses still stand; that is, paraoxon is the principal toxic constituent of the residue and the exposure is dermal via the fallout mechanism. This year we used positive pressure half-mask respirators fed with air filtered through activated charcoal and pumped into the mask by a back-pack unit. Again, no differences could be detected in cholinesterase response between those with and those without respirators. In fact, during one exposure sequence of 12 hours, one man wearing a respirator unit showed a 27% decline in RBC cholinesterase, which was the greatest decline of any member of the crew during that period.

The next facet of our work will be to ascertain if the levels of the oxygen analogs of the organophosphate pesticides are of similar importance to the residue poisoning problem in regions of the country other than the Central Valley of California. The suggestion that these metabolites were central to the problem was made by Milby et al. in 1963 but their clue was not pursued until quite recently. If our findings are confirmed and a reliable correlation established between residue level and biological response, substantial progress will have been made toward the resolution of this occupational health problem.

ACKNOWLEDGEMENT

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PRELIMINARY STUDIES OF ASBESTIFORM FIBERS IN
DOMESTIC WATER SUPPLIES

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INTRODUCTION

It has been evident for some years that there exists a strong positive association between inhaled asbestos fibers and carcinoma of the lung (NIOSH, 1972; Selikoff, 1965 and 1967). This association has been particularly notable in occupational groups such as insulation workers employed in industrial environments having high concentrations of asbestos fibers. An even more dramatic phenomenon is the increased number of rare pleural mesotheliomas among these workers. Selikoff (1973) has presented data which indicate that the incidence of some abdominal cancers, including peritoneal mesothelioma, is also greatly increased (more than three-fold) among asbestos workers.

If asbestos fibers are carcinogenic, they may well be expected to act as carcinogens by various routes of entry into the body, including the oral route. Recently, Pontefract and Cunningham (1973) presented data which gave some evidence that asbestos fibers pass through the gut and into the organs of experimental animals.

A potential source of exposure to asbestos fibers which could be ingested is through municipal drinking water supplies. Such supplies may be contaminated with asbestos from industrial operations or naturally in supplies which are collected from and maintained in reservoirs and aquifers which are exposed to serpentine or other geologic formations which contain asbestos.

The presence of asbestos fibers in drinking water supplies, located in Ottawa and Montreal, Canada, has been reported by Cunningham and Pontefract in 1971. In this instance filtered water was reported to contain from 2.0×10^6 to 5.9×10^6 asbestos fibers per liter and unfiltered (raw) water was proportionately higher. More recently, Cook et al. (1974) have reported the presence of 1 to 30 million amphibole asbestos fibers per liter in the city of Duluth water supply. In this latter instance the source of the asbestos was from a nearby milling operation.

In this paper we wish to report upon the results obtained in the analysis of numerous drinking water sources for asbestiform fibers in the United States, with emphasis on Northern California. In this effort we have developed a technique for asbestos analysis which seems to compare favorably with the results of others who are using a similar methodology. The development of an acceptable standard method of analysis is essential to the conduct of a rational epidemiological assessment of the impact of asbestiform fibers in drinking water supplies upon human health.

METHODS

Samples were collected from the various water sources by the authors, representatives of the Environmental Protection Agency, or by employees of the various water districts involved. All of these were grab samples ranging from 1 to 4 liters in volume.

The method of analysis for the presence of asbestos fibers required concentration of the sample by filtration and the examination of the concentrate using the Siemens Elmiskop I or IA Electron Microscope. A measured amount of sample was filtered through a sequence of 0.47 mm diameter, 0.8 μ and 0.2 μ pore size, polycarbonate Nuclepore filters (Nuclepore Corporation, Pleasanton, California). Both filter sizes were necessary in order to facilitate filter rates and to capture small fibers. The ultimate amount of water filtered was proportional to the original turbidity of the collected samples; normally from 300 to 1000 ml of sample were filtered.

A pseudo-replica was made of the particulates concentrated on the filters by coating them with silicon monoxide in a high-vacuum optical coater. A 1/8 inch diameter disc was cut from the coated filter with a sharp boring tool and mounted, coated side down, on a 200-mesh nickel electron microscope grid previously placed on top of a block of polyurethane soaked in chloroform. A small petri dish is used to hold the polyurethane and chloroform. The Nuclepore filter is dissolved by the chloroform by wick action through the pores of the SiO replica on the filter surface. The replica is left on the grid with the particles and fibers on top of the SiO substrate. The latter process takes from 4 to 6 hours. At the end of this time, the prepared grids are ready for electron microscopy.

The Siemens Elmiskop Electron Microscope was operated at 100 KV. The fibers were counted and sized directly on the microscope viewing screen on which was ruled a 1 cm² grid with 2 mm intervals. The magnification used for sizing and counting was 2000 and/or 20,000X. For identification of fibers by morphology and electron diffraction, the magnification used was 20,000X. At this magnification selected area diffraction can be used to obtain the electron diffraction pattern of a crystal or group of crystals. This pattern is usually a spot pattern which is characteristic of various types of material.

Fibers were counted and recorded for each of 5 to 20 fields in a 200-mesh specimen grid. A field is defined as a single opening in the specimen grid. The ratio of the total number of fields in a grid to the number of fields counted is used in the calculation of the number of fibers on the whole grid. The total number of openings in a grid was determined using a photographic enlargement of an electron microscope grid. A fiber was defined as a particle whose length was at least 3 times its diameter.

RESULTS

Using the described method 19 samples of water from California, 5 from Houston, Texas, 1 from Wisconsin, and 9 from Duluth, Minnesota, were examined for their asbestiform fiber content. The results of these examinations are shown in Tables 1 and 2.

TABLE 1. ASBESTOS FIBER ANALYSIS OF VARIOUS WATER SOURCES
IN CALIFORNIA

<u>Geographic Area</u>	<u>Sample Date</u>	<u>Sample Source</u>	<u>Treatment</u>	<u>Fiber Count Millions of fibers/liter Chrysotile</u>
Marin Co.	2/73	Lake A (Rainy Season)	Raw	200
Marin Co.	2/73	Stream into Lake B (Rainy Season)	Raw	Present ⁽¹⁾
Marin Co.	2/73	Lake C (Rainy Season)	Raw	Present
Marin Co.	2/73	Lake A (Rainy Season)	Coag., filtered	Not analyzed due to pres- ence of colloid
Marin Co.	7/73	Lake A (Dry Season)	Raw	0.3
Marin Co.	7/73	Lakes A & B (Dry Season)	Raw	0.5
San Francisco Co.	7/73	Office Bldg. Tap (from Crystal Springs Res.; Hetch Hetchy Syst)	Not Coag., not filtered	1.0
San Francisco Co.	7/74	Office Bldg. Tap (from Crystal Springs Res.; Hetch Hetchy Syst)	Not Coag., not filtered	0.2

TABLE 1 (Continued)

<u>Geographic Area</u>	<u>Sample Date</u>	<u>Sample Source</u>	<u>Treatment</u>	<u>Fiber Count Millions of fibers/liter Chrysotile</u>
Alameda Co.	7/74	Filter Plant Effluent	Coag., filtered	---
Alameda Co.	7/74	Treatment Plant Effluent	Not Coag., not filtered	---
Contra Costa Co.	7/74	Filter Plant Effluent	Coag., filtered	---
Burlingame City	7/74	Tap-lateral Hetch Hetchy, Crystal Springs	Not Coag., not filtered	---(2)
Redwood City	7/74	Tap-Calavaras Lake (Surface and/or Hetch Hetchy), infiltration gallery	Not Coag., not filtered	---
Millbrae City	7/74	Tap-San Andreas Res, local surface water	Not Coag., not filtered	---
Lawrence Livermore Laboratory	5/74	Tap in Lab., Hetch Hetchy before Crystal Springs	Not Coag., not filtered	---
San Jose City	7/74	Tap-groundwater	Not Coag., not filtered	---
Southern Calif.	7/74	Effluent-Filter Plant A, SWP(3)	Coag., filtered	---
Southern Calif.	7/74	Effluent-Filter Plant B, CRA(4)	Coag., filtered	---
Southern Calif.	7/74	Effluent-Filter Plant C, CRA	Coag., filtered	---

(1) Fibers could not be counted because of gross amounts of turbidity present in sample.

(2) Fibers not detected.

(3) Water originates from the California State Water Project.

(4) Water originates from the Colorado River.

TABLE 2. ASBESTOS FIBER ANALYSIS OF VARIOUS WATER SOURCES OUTSIDE OF CALIFORNIA

<u>Geographic Area</u>	<u>Sample Date</u>	<u>Sample Source</u>	<u>Treatment</u>	<u>Fiber Count</u> Millions of fibers/liter Amphibole
Houston, Texas	7/73	Water Syst. A groundwater		---(1)
Houston, Texas	7/73	Water Syst. B groundwater		---
Houston, Texas	7/73	Water Syst. C groundwater		---
Houston, Texas	7/73	Water Syst. D groundwater		---
Houston, Texas	7/73	Water Syst. E groundwater		---
Silver Bay, Minnesota	7/73	Lake Superior	Not Coag., not filtered	2
Beaver Bay, Minnesota	7/73	Lake Superior	Not Coag., not filtered	3
Two Harbors, Minnesota	7/73	Lake Superior	Not. Coag., not filtered	2.5
Duluth, Minnesota	7/73	Lake Superior, Dist. Syst. 1	Not Coag., not filtered	5
Duluth, Minnesota	7/73	Lake Superior Dist. Syst. 2	Not Coag., not filtered	2
Duluth, Minnesota	7/73	Lake Superior Dist. Syst. 3	Not Coag., not filtered	1
Duluth, Minnesota	7/73	Lake Superior Dist. Syst. 4	Not Coag., not filtered	1
Duluth, Minnesota	4/74	Lake Superior spring turnover	Not Coag., not filtered	10

TABLE 2 (Continued)

<u>Geographic Area</u>	<u>Sample Date</u>	<u>Sample Source</u>	<u>Treatment</u>	<u>Fiber Count Millions of fibers/liter Amphibole</u>
Duluth, Minnesota	4/74	Lake Superior spring turnover	Not Coag., not filtered	8
Ashland, Wisconsin	7/73	Groundwater	Not Coag., not filtered	---

(1) No fibers detected.

Samples of water taken from the San Francisco Bay Area (Marin Co., San Francisco Co., Alameda Co., Burlingame City, Redwood City, Millbrae City, San Jose City, and the Lawrence Livermore Laboratory; see Figure 1) originate from sources both indigenous to the area and from sources outside the area. The Marin County water source is from local runoff collected in lakes located in areas which include a good deal of sheared (friable) serpentine rock. These waters contained from 300,000 to 200 million chrysotile fibers per liter, the latter in the dry season and the former during the wet time of the year. The majority of San Francisco County water originates in the granitic Sierra Nevada Mountains near Yosemite (Hetch Hetchy Reservoir) and is stored, along with local water, in the Crystal Springs Reservoir. This latter lake is situated in a serpentine area.

Only 2 samples were taken from the San Francisco system, and these were taken a year apart (both during the dry season). The first sample contained 1 million fibers per liter while the subsequent sample was an order of magnitude less. A good deal of highway construction was underway along the edge of this lake from 1965 until early 1974, and the occurrence of these chrysotile fibers may well have resulted from the disturbance caused by the construction effort. It is interesting to note that the Lawrence Livermore Laboratory water, the source of which is Hetch Hetchy water before it reaches Crystal Springs, had no detectable fibers present ($< 1 \times 10^4$ fibers/liter). It is also of interest that Millbrae City water, which originates in the San Andreas Reservoir located just north of Crystal Springs (See Figure 1), contained no detectable asbestiform fibers ($< 2 \times 10^4$ fibers/liter). In this instance the local geological formation is more Franciscan than ultrabasic (just the reverse of Crystal Springs), as one might suspect a less pronounced source of chrysotile asbestos fibers. No fibers were detected ($< 2 \times 10^4$ fibers/liter) in either Redwood City or the City of Burlingame's water supply. Both these districts receive Hetch Hetchy water, and the latter may or may not receive Crystal Springs water depending upon the operational mode of the system.

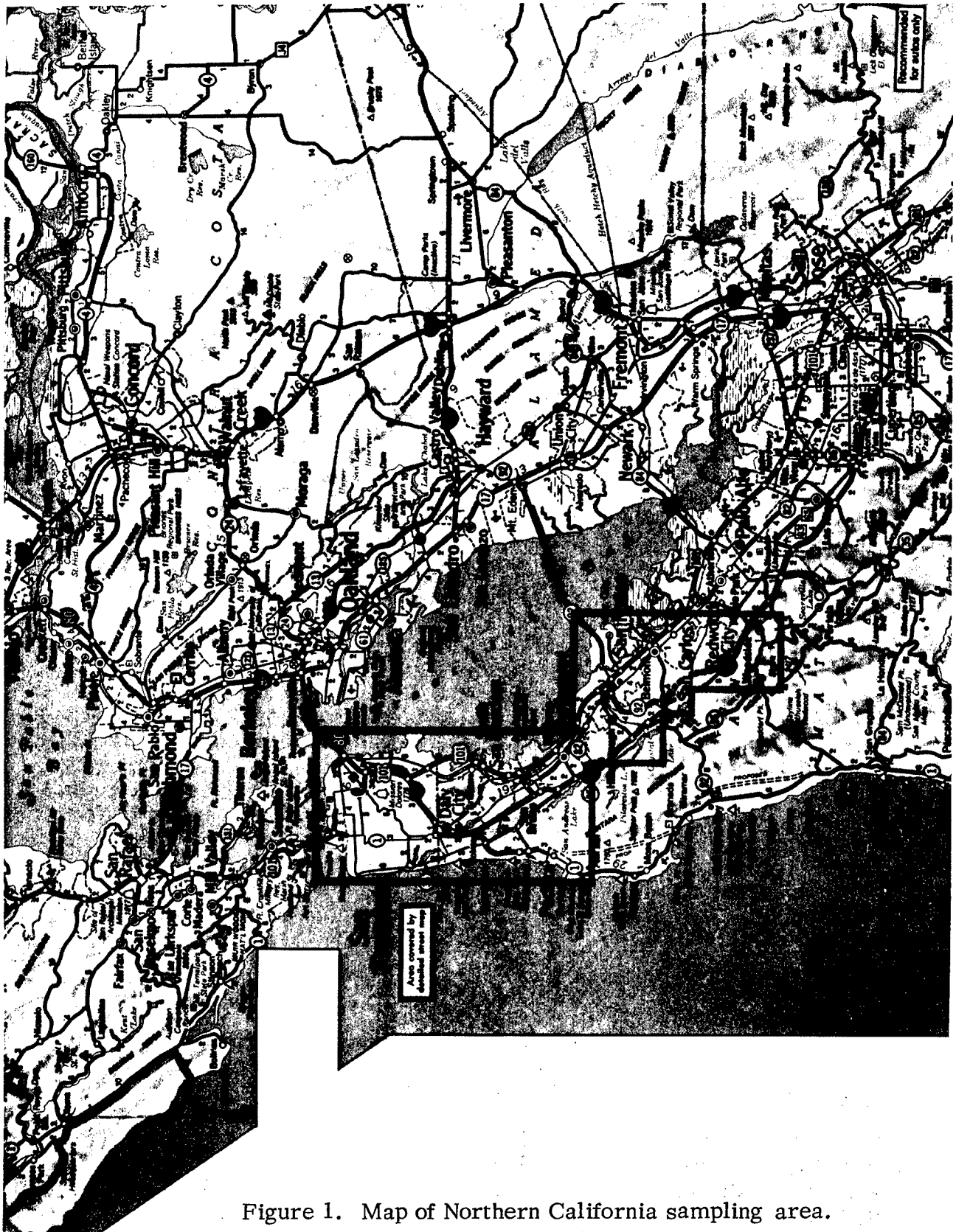


Figure 1. Map of Northern California sampling area.

San Jose City water supply is from a local groundwater source and no fibers were detected ($<1 \times 10^4$ fibers/liter). Water samples from Alameda and Contra Costa Counties (San Francisco East Bay Area) contained no detectable fibers ($<1 \times 10^4$ to 4×10^4 fibers/liter). None of the sources are associated with obvious serpentine formations. The samples from Southern California were from either the State Water Project or from the Colorado River and had all received complete water treatment including coagulation and filtration; these were all negative (<1 to 2×10^4 fibers/liter).

Results from samples taken from areas outside of California are shown in Table 2. Groundwater from Houston, Texas and from Ashland, Wisconsin were found to be negative (<1 to 4×10^4 fibers/liter). All the samples originating in Lake Superior (Silver Bay, Beaver Bay, and Two Harbors) and those used in the Duluth water system were found to be positive ranging from 1×10^6 to 10×10^6 fibers per liter. All of these fibers were identified as amphibole asbestos (Grunerite-Cummingtonite). Eighty-five percent of these fibers ranged from 0.1 to 0.5μ in diameter by 0.5 to 3.0μ in length. The remainder were 0.5 to 1.0μ in diameter by 4.0 to 7.0μ in length. These originated from Taconite milling operations about 40 miles northeast of Duluth.

The asbestos found in California waters was identified as chrysotile with fibers 2 to 10μ long and about 0.03μ in diameter for single fibers and bundles of fibers 0.5 to 1.0μ in diameter (see Figures 2 and 3 for examples of these fibers seen in water).

The results obtained using the electron microscope method herein described appear compatible with the results reported from Lake Superior by Cook et al. (1974) who used a different method of sample preparation. We also had the opportunity to analyze samples collected by the Environmental Protection Agency which were simultaneously analyzed by Mr. Ian Stewart of McCrone Associates, Inc. (Chicago, Illinois). The latter used a method similar to ours except that a millipore filter was used (Millipore Filter Corporation, Massachusetts) to concentrate the sample and a collodion-covered, carbon-coated grid was used for the pseudo-replicate and the membrane filter dissolved with acetone in a soxhlet apparatus. The comparative results are shown in Table 3.

TABLE 3. COMPARISON OF ANALYTICAL RESULTS
OBTAINED BY TWO LABORATORIES USING VARIATIONS
OF THE ELECTRON MICROSCOPIC METHOD

<u>Water Source</u>	<u>Univ. of Calif. Method*</u>	<u>McCrone Method*</u>
Silver Bay, Minnesota	2.0	4.4
Beaver Bay, Minnesota	3.0	5.3
Two Harbors, Minnesota	2.0	2.5
Duluth, Minnesota	5.0	2.8
Houston, Texas	None detected	None detected

*Measured in million fibers per liter.



Figure 2. Electron photomicrograph of chrysotile asbestos fiber found in a Northern California raw water supply.



Figure 3. Electron photomicrograph of amphibole asbestos fibers found in Lake Superior water.

As can be seen in Table 3, there is good agreement between the two laboratories.

DISCUSSION

The method developed in our laboratory for the electron microscopic analysis for asbestiform fibers in natural waters appears to be a satisfactory analytical tool. The results obtained using this method agree reasonably with the results of analyses reported by others using variations of the electron microscopic approach. We feel it is preferable to the other methods thus far developed because it is the simplest to perform and disturbs the sample less than methods which require ashing, sonication, and/or centrifugation prior to enumeration. Light microscopic techniques, such as are often used in the analysis of air for fibrous material, are unsatisfactory in the analysis of water for these substances because of the relatively large amounts of extraneous material present. With the electron microscope, even used at light microscope magnification levels, the resolution is much greater, which is important in defining the morphology of fibers particularly when they are mixed with a variety of debris and microplankton. Also, the capability to obtain selected area electron diffraction patterns for crystal identification is not available with the light microscope.

The major limitation of all the methods presently employed is the restricted volume of water which can be filtered through fine-pore filters within a reasonable time. Because of the frequent presence of much extraneous matter, particularly in raw water, the technician is usually limited to processing a liter or less. This, of course, in turn limits the sensitivity of detection of fibers to no less than 100,000 fibers per liter of the original sample.

The results of our analysis of waters from various areas of the country are certainly preliminary. The numbers of samples taken from each supply have been limited and these have been isolated (grab) samples only. It would be of far more value if these samples were composited over time and we hope that funds will become available to allow such sampling of selected supplies. However, even with these imposed limitations, the results obtained have been most instructive of the distribution and types of asbestiform fibers found in water supplies. The samples taken from Lake Superior in the Duluth, Minnesota region contained fibers identified as amphibole asbestos, whose origin was from an industrial operation. The numbers we found were similar to those reported by Cook et al. (1974). We became interested in the potential introduction of asbestos fibers from natural sources, prior to the national concern about the Duluth situation, when we became aware that certain water impoundments were located in areas which contained large outcroppings of serpentine rock. Our analytical results from the various Northern California water supplies certainly indicate that water thus exposed can contain considerable amounts of chrysotile asbestos

fibers, equivalent or even greater in number than amounts of amphibole fibers found in the Duluth area. Thus, if asbestiform fibers are a problem in water supplies, it is not a problem brought about solely by pollution of water sources but can arise from contact with natural geologic formations as well.

It appears from our data that, as stated above, one can predict the occurrence of these materials by understanding the geology of the watershed. It also was seen that such geologic diagnosis should be made locally and on-site since even the water of two adjacent but separate reservoirs (Crystal Springs and San Andreas Lake in San Francisco County) were exposed to different geologic formations and such exposure was reflected in the fiber content found in each of these waters. The geologic difference may not be recognized from small maps of the general area.

No fibers were detected in any of the groundwater samples collected. We were able to analyze some fully treated waters (coagulated and filtered) and could detect no fibers. However, in the case of the Southern California samples, we have not yet been able to perform the analysis of the raw water prior to treatment and in the case of the Marin County treated water there were technical difficulties with colloidal material in the finished water which made particle analysis impossible. Thus, no statement as to the ability of water treatment processes to remove asbestiform fibers can be made.

It would be premature to draw conclusions concerning the impact upon the public health of exposure of large populations to asbestiform fibers through water supplies. The serious impact of industrial exposure is clear and by inference gives us cause for concern regarding the presence of asbestos in water supplies. Information concerning the efficacy of water treatment processes in the removal of these fibers is urgently needed as well as epidemiological studies of populations exposed to water supplies containing asbestiform fibers in the amounts thus far detected.

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WATER REUSE IN THE UNITED STATES

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Thank you Mr. Chairman, ladies and gentlemen. I understand that the opening session of this conference represents something of an innovation with the introduction of a discussion of Environmental Quality: Evaluation and Research. This is most fitting and appropriate since this is the Fifth Annual Conference on Environmental Toxicology sponsored by the Aerospace Medical Division of the USAF and directed towards a discussion of traditional areas of mammalian toxicology. I am happy and privileged to have been invited to address you in an area of environmental quality which is taking on greater significance each day. This is the area of water reuse. I shall direct my remarks more particularly to water reuse in the United States.

"Water, water, everywhere, nor any drop to drink." When Coleridge first authored these famous words during the early nineteenth century he was naturally referring to the ocean, since at that time the concept of water pollution must have been almost unknown. Today water pollution is not only well established, but it has become an alarming reality which could, if not checked in time, obtain a stranglehold on the social and economic progress in industrialized countries.

The demand for water is increasing both through population growth and changing life styles brought about by advancing technology, while the supply of water from nature remains basically constant from year to year. This is not to imply that we are or will shortly be out of water, although water shortages are of great concern in some areas. Rather, we must recognize the potential for the use and reuse of wastewater where possible.

There is a growing interest in wastewater reuse in the United States. This is particularly true in water short areas of increasing water demand resulting in depletion of good quality natural water sources.

With the increased level of indirect water reuse occurring in this country, and an expanding pressure for direct potable reuse of wastewater, many of the active organizations concerned such as the American Water Works Association and the Water Pollution Control Federation are now addressing themselves to this problem in an official way through the formation of a joint committee. In addition there are groups within certain Federal Agencies, such as the EPA Water Supply Division that are now turning their efforts towards the area of water reuse.

Consensus among individuals in the fields of public health, wastewater utility management, engineering, and the university sectors as laid out by the WPCF -AWWA Joint Committee is that the following represent the more pertinent aspects of water reuse:

1. Ever greater amounts of treated wastewaters are being charged to the waters of the nation and constitute an increasing proportion of many existing water supplies.
2. A large number of proposals are being made to introduce reclaimed wastewaters directly into various elements of domestic water supply systems.
3. Sound management of our total available water resources must include consideration of the potential use of properly-treated wastewaters as part of drinking water supplies.
4. There is insufficient scientific information about acute and long-term effects on man's health resulting from such uses of wastewaters.
5. Fail-safe technology to assure the removal of all potentially harmful substances from wastewaters is not available.
6. Federal support is required for an immediate and sustained multidisciplinary national research effort to provide the scientific knowledge and technology relative to water reuse to assure the protection of public health.

Effective water management seeks to assure that adequate quantities of good quality water are available when needed and where they are needed, at the lowest possible cost. Increased public pressure for more effective pollution control measures over the past decade has served to stimulate reuse of municipal and industrial, agricultural and recreational uses. Water managers faced with the increased cost of improving the quality of wastewater effluents are finding reuse to be an economic alternative to increasing water supply capacity for industrial process and cooling needs. Current public pressure for advanced waste treatment to further improve effluent quality is stimulating more serious discussion of direct reuse for human consumption. This is the ultimate concern of the Water Supply Programs Division of EPA: that is the public health considerations associated with direct reuse of treated wastewater for domestic supply.

The Water Supply Division was transferred from the Public Health Service, DHEW when EPA was formed in December 1970. The then existing authorities and responsibilities of the Division were also transferred. The activities of the Division can be summarized briefly as follows:

The Division is active in six water-related fields of endeavor. There are programs under way to update the Drinking Water Standards, carry out the provisions of the Interstate Quarantine Regulations with regard to interstate carrier water supplies, and provide an effective basic and applied research program on health effects of intimate contact with water; also to provide specialized technical services regarding public drinking water supplies, advance and implement public health and water supply aspects of water resources and provide consultation on the health aspects of water pollution.

Reuse is usually referred to as "direct" or "indirect." For the purposes of this paper, direct reuse is accomplished by recycling waste treatment plant effluents directly to the water treatment plant. Indirect reuse involves the discharge of effluents to bodies or watersheds where it becomes available for use again.

In a recent article, Wolf (1971) observed that the concept of wastewater reuse is not new. People have long been aware that some cities discharge sewage to water sources upstream of other cities' water supply intakes. He cites the extreme case of emergency reuse at Chanute, Kansas in 1956 and 1957 where chlorinated secondary treatment plant effluent was recycled through a dried up river bed back to the city's water intake. The process was primitive by today's technology and the product was objectionable due to turbidity, odor-taste and foaming, but the water reportedly contained no detectable pathogenic organisms. However, other reports indicate that the citizens used bottled water and other sources for drinking.

McCallum (1962) reported the concept of direct wastewater reuse to be technically and economically attainable and he urged engineers, chemists and bioscientists to work together to make it so. He may have overlooked some of the pertinent public health considerations involved or may have been premature in his assessment at that time because Berg (1971) seemed to disagree. Discussing the transmission of virus to humans by the water route he says, "Investigation of the problems of viruses in water is in its infancy. The major problems of what waterborne viruses are important to us, how they can be quantitatively detected and identified in waters of all qualities, how effectively treatment processes remove them, and how they can be destroyed in waters of all qualities are still unanswered. The resolution of all of these problems will mark the direction of research in this area for the next decade."

In March, 1973 the Environmental Protection Agency issued its policy statement on water reuse which may be summarized as follows:

1. The Environmental Protection Agency supports and encourages the development and practices of successive wastewater reclamation, reuse, recycling and recharge as a major element in water quality management, providing the reclamation systems are designed and operated so as to avoid health hazards to the people or damage to the environment.
2. EPA recognizes and supports the potential for wastewater reuse in agriculture, industrial, municipal, recreational and groundwater recharge applications.
3. EPA does not support the direct interconnection of wastewater reclamation plants with municipal water treatment plants.
4. EPA not only is continuing to support reuse research and demonstration projects, but now has underway a research program covering a wide spectrum of health effects research in this area.

In 1971, the American Water Works Association made public its views in the following policy statement:

The Association believes that the full potential of reclaimed water as a resource should be exploited as rapidly as scientific knowledge and technology will allow, to the maximum degree consistent with the over-riding imperative of full protection of

the health of the public and the assurance of wholesome and potable water supplied for domestic use. The Association encourages an increase in the use of reclaimed wastewaters for beneficial purposes, such as industrial cooling and processing, irrigation of crops, recreation and within the limits of historical practice, groundwater recharge. Further, the Association commends efforts that are being made to upgrade wastewater treatment, and to improve quality before discharge into sources of public water supplies.

The Association is of the opinion, however, that current scientific knowledge and technology in the field of wastewater treatment are not sufficiently advanced to permit direct use of treated wastewaters as a source of public water supply and it notes with concern current proposals to significantly increase both indirect and direct use of treated wastewaters for such purposes. It urges, therefore, that immediate steps be taken, through intensive research and development ... to advance technological capability to reclaim wastewaters for all beneficial uses. Such research and development is considered to be of greater national need than that now being directed to desalinization. It should:

1. Identify the full range of contaminants possibly present in treated wastewaters which might affect the safety of public health, the palatability of the water and the range of concentrations.
2. Determine the degree to which these contaminants are removed by various types and levels of treatment.
3. Determine the long-range physiological effects of continued use of reclaimed wastewater, with various levels of treatment, as the partial or sole source of drinking water.
4. Define the parameters, testing procedures, analytical methodology, allowable limits, and monitoring systems which should be employed with respect to the use of reclaimed wastewaters for public water supply purposes.

5. Develop greater capability and reliability of treatment processes and equipment to produce reclaimed water of reasonably uniform quality in view of the extreme variability in the characteristics of untreated wastewaters.
6. Improve the capabilities of operational personnel.

The concern expressed here is valid from a public health standpoint. Fundamental to the problem is the fact that, with existing technology, there is no economical process available to remove all contaminants in a single step. Wolf (1971) observes that such a degree of treatment, to remove "everything," is not being proposed because economic considerations make it impractical.

We repeatedly see references to articles proclaiming that sewage has been treated to such a high degree that it meets Drinking Water Standards. Wolf condemns such statements as inaccurate. He points out that what is meant is that they meet "some of the requirements of the Drinking Water Standards." There are two paragraphs (the first two definitions, in fact) in the Drinking Water Standards that are very much a part of those Standards. The requirements set up in these paragraphs must be met in order to render the judgment that a specific product water "meets Drinking Water Standards." The first refers to "adequate protection by natural means" which "involves one or more of the following processes of nature that produces water consistently meeting the requirements of these Standards: dilution, storage, sedimentation, sunlight, aeration, and the associated physical and biological processes which tend to accomplish natural purification in surface waters, the natural purification of water by infiltration through soil and percolation through underlying material and storage below the groundwater table."

The second paragraph is concerned with "adequate protection by treatment" which means "any one or any combination of the control processes of coagulation, sedimentation, absorption, filtration, disinfection, or other processes which produce a water consistently meeting the requirements of these Standards. This protection also includes processes which are appropriate to the source of supply; works which are of adequate capacity to meet maximum demands without creating health hazards, and which are located, designed and constructed to eliminate or prevent pollution; and conscientious operation by well-trained and competent personnel whose qualifications are commensurate with the responsibilities of the position and acceptable to the Reporting Agency and the Certifying Authority."

Since "adequate protection by treatment" has not been defined by EPA, which is the Certifying Authority referred to, there are no such waters that "meet Drinking Water Standards." It can, therefore, be stated that the Standard is also similarly questionable if it is to apply to a source water when that source water is sewage.

The 1971 recommendation of EPA's technical task force on the revision of the Drinking Water Standards states in part that, "These Standards apply to a water supply that has been obtained from the most desirable source and are not intended for application to wastewaters effluent, used directly as a raw water source." We must remember that the Drinking Water Standards were developed to be used in connection with a sanitary survey which would assure that the source was relatively unpolluted. The Standards have never taken into consideration the possible toxic elements and compounds likely to be found in wastewaters.

In the field of public health engineering two principles are fundamental:

1. Innovation must be considered suspect until proven safe. We must assume that the reuse of wastewater for human consumption poses health hazards until proven otherwise.
2. Prevention of illness or disease, not the cure, is the objective. Borderline "safe" operations are not acceptable; there must be adequate margins of safety continuously maintained. For each innovation, there must be thorough pilot and field testing demonstrations, development of failsafe monitoring techniques and controls and provision of standby equipment and procedures.

The classic example cited by advocates of direct reuse for potable water supply is the facility in operation at Windhoek, South-West Africa. Effluent from a 1 mgd advanced waste treatment plant is directly recycled for human consumption. The successes in South Africa have received international acclaim. However, it is significant to note that the top scientists in South Africa in the fields of virology, bacteriology and chemistry have complete control over the operation of the facility. Public utilities in the United States cannot afford the use of full time expertise of that stature.

Long and Bell (1972) outline some of the key health factors to be considered which include viruses, bacteria, other microorganisms, chemicals and the reliability of plant operation. They point out that the endemic threat of viruses derives from certain key factors:

- 1) The infectious dose of viruses is very low; recent studies using vaccine strains of poliovirus, indicated that one Plaque Forming Unit (PFU) taken orally would infect 30% of the experimental group.
- 2) Clinical illness is observed in only a small fraction of those who become infected; the ratio of clinical cases to subclinical infection ranges from 1:10 to 1:1000 depending on the species of virus involved. Subclinical infections of enteric viruses in man cannot be considered as innocuous to health; in addition, certain enteric viruses are associated with serious delayed effects on the infected persons or even their children, Coxsackie viruses have been incriminated as teratogenic agents and the affinity of these viruses to heart tissue has become increasingly recognized from experimental studies as well as clinical and epidemiological observations.
- 3) One strain of virus may produce illnesses with widely different incubation periods and clinical manifestations; for instance, a Coxsackie virus may produce meningitis in one person, myocarditis in another and diarrhea in a third. Obviously, this factor would additionally complicate the epidemiological identification of waterborne source of viral infection.
- 4) The killing rate of chlorine for different viruses is variable and generally much slower than for bacteria. Recent work by Liu (1971) of the Water Supply Research Laboratory has demonstrated this variability and has also provided leads for other interesting questions on virus behavior. One experiment showed that, for the same degree of kill with the same concentration of chlorine it took 2.7 minutes for a reovirus and 36.5 minutes for the Type II poliovirus. Liu (1971) has also shown that the rate of inactivation for several viruses decreased during the latter part of the inactivation period; theoretically this may be related to the occurrence of virus-clumps or viruses absorbed on particles which would be a likely occurrence in nature (wastewater) and would appear to be more resistant to inactivation.

Against this variability background for inactivation by chlorination and the low infectious dose rate, together with the physiological factors of subclinical infection and pathological variations resulting from the same viral exposure, it can be seen that considerable health damage may be occurring from viruses in drinking water without being readily identified. We call this the "ripple" effect.

Long and Bell (1972) illustrate the point with the following hypothetical situation:

- A tertiary waste treatment plant with 99% virus reduction discharges one to seven virus units per 100 ml.
- With adequate chlorination for a 99.99% reduction of virus, there still is at least one PFU per 50 gallons (or 1×10^6 units in a 50 MGD supply).
- This is the kind of supply many advocate for reuse with no additional treatment.
- With 0.2% of the total supply ingested as drinking water - and a 30% infection rate.
- Up to 600 people would be impacted with a variety of clinical and subclinical infections daily.
- If they in turn shed their viruses through personal contact throughout the community, each case can create a "ripple" affecting many people through a "non-water" route.
- The actual waterborne infection being no more of an order-of-magnitude than the tip of an iceberg represents the whole.

The problem of viruses is especially difficult to deal with since their action is so insidious and the ramifications of their subclinical damages are so pervasive. Much of the chronic ill-health in the nation may be related to low viral infectivity and water is a potential prime route for such infection.

These facts and logical speculations give rise to concern not only for the direct reuse of renovated wastewater but also for the effectiveness and safety of current practices for treating and delivering drinking water.

A second group of concern is bacteria. Examination and extrapolation from data obtained on fecal coli and streptococci in advanced waste treatment studies shows that treatment prior to chlorination should remove salmonellae and other enteric pathogenic bacteria to the same extent that viruses are removed; further that subsequent chlorination will be more effective in destroying these bacteria than the viruses.

However, unusual circumstances and breakdowns can occur and bacteria may not be so simply handled. Recent studies of hospital infection outbreaks have demonstrated that non-enteric bacteria can, under certain circumstances and in sufficient concentrations, present health hazards. Further, two types of these bacteria, the flavobacteria, (involved in four cases of septicemia following open-heart surgery) appear to be resistant to chlorination and can multiply greatly, given time and an appropriate medium.

When these findings are joined with the knowledge that potential elements of advanced wastewater treatment processes may harbor and facilitate the multiplication of bacteria and that recycling may serve to preserve and increase the bacterial seed, one has reason to investigate and handle bacterial aspects with caution. For example, bacterial growth occurs on dialysis and osmosis membranes, ion exchange columns and carbon columns; if these growths should include colonies of chlorine-resistant bacteria such as flavobacteria, then the reclamation plant and the reuse process could serve as a promoter of these infectious agents rather than as a barrier to them.

A variety of other organisms occur in wastewaters and can cause health damage. Prominent among these are protozoa, some of which are resistant to chlorination and have been implicated in the parasitization of the throats of children, and in cases of cerebromeningitis. Free nematodes have been found in municipal water supplies and can serve as carriers for enteroviruses, salmonellae and shigellae.

Lastly, helminth infections have been spread by the practice of utilizing wastewaters for the watering of gardens and lawns; ascaris, trichuris and even hookworm infection may be spread in this manner. Severe and extensive infection of ascaris and trichuris occurred in several European countries in the post World War II years from irrigation of vegetable gardens with sewage effluent; the residents of Munich, Germany were hit worst. Effluent irrigation of pastures has also been shown to infect cattle with beef tapeworm, which in turn, could infect man.

Studies relating to the health aspects of wastewater reuse should consider helminth infections in recreational bathing projects and both protozoan and helminth infections in reuse for the irrigation or processing of food crops.

Chemicals in drinking waters may exert acute or chronic effects on humans. We all are aware that the home today is a veritable chemical depository, and the sewer in turn acts as a ready receptacle for disposal of all these materials. In addition, a great variety of activities within the community contribute to a considerable degree to the use of the sewer as a chemical dumping ground. When we consider the universal availability of many highly toxic substances, some concern should also be directed towards the possibility of deliberate sabotage or accident to our water supplies. Some thought should be given to a holding period prior to reuse along with a continuous monitoring program which includes some type of bioassay of the renovated waters. Without this, any immediate and direct reuse of renovated wastewaters will continuously expose the public to hazards from accidental or malicious spilling of toxic chemicals into the sewer system. The extent of the problem can be partially demonstrated by using actual reported data for toxic chemicals entering the Ohio River Basin since 1969. Over 2 million gallons of such exotic chemicals as vinyl cyanide (3,800 gallons), phenol (2,700 gallons), 2-4 toluene di-isocyanate (11,000 lbs), and 17,500 pounds of "assorted organic chemicals" entered the river. Similar spills (and dumps) obviously occur in many cities and reach the sewage treatment plants and pass through such process unaltered. Most treatment processes in use today have little effect on the removal of these chemicals.

In the case of the disposal of chemicals via the sewer as compared with that dumped into rivers, the volumes of contaminants may not be as great but neither is the dilution factor. It is currently estimated that only 20% of the spills of toxic chemicals are even reported, and the time period for reported spills has ranged from 1 up to an incredible 36 days during 1971.

Most of the detailed studies of the chronic health effects due to the many chemicals currently found in waters now used by the American consumer have yet to be accomplished, and there is practically no information available on the health hazards of chemicals found in renovated wastewaters. Among the inorganics: mercury, lead, arsenic, chromium, cadmium, nitrate, and cyanide are of extreme importance and have been addressed in the 1962, as well as the 1974 Drinking Water Standards. In addition, many others such as sodium are important to those whose marginal state of health requires certain additional consideration and concern in contemplating the direct use of renovated wastewaters. Among the organics: o-nitrochloro benzene, pyridine, chlorinated pesticides, diphenyl ether, kerosene, nitriles, benzene derivatives, and many others are of extreme concern and require extensive research. Many newer pesticides and herbicides have been added to the list of potentially hazardous chemical pollutants.

Long and Bell (1972) cite a recent report from the Netherlands indicating the formation of a toxic product, methionine sulfoxide, when chlorine is added to water containing ammonia or amino groups and methionine (a compound found in flour mill waste).

Among other chemical aspects requiring attention are such organic compounds as those containing a benzpyrene base and known to be carcinogenic. In addition, potential sex hormone effects resulting from the increasing use of birth-control pills and their residue or by-products which may be returned to sewer systems could also present a problem. Research on wastewater reuse should include characterization and toxicity studies of the recovered organics by carbon filtration, as well as the development of a method or methods to recover those organics that are not removed by carbon absorption. Studies concerned with the identification of these compounds and the determination of their chronic toxicity at low levels and/or their carcinogenic risk are essential. Such studies have hardly commenced as yet. This information is needed in order to develop specific MAL's for these materials.

It is certainly obvious that prior to the intimate use of any wastewater, the chemical characteristics of that wastewater must be determined and adequately monitored in order to assure that no substance of a harmful nature reaches the ultimate user.

In spite of the extensive process developed by AWT facilities thus far, the fact remains that there are harmful metals and organics that will pass through such processes.

In depth toxicological testing of highly treated sewage effluents has not been accomplished; therefore we are not in a position to completely and safely judge the health implications. When such knowledge does become available, we still must develop systems to continuously monitor for these substances.

For the most part, non-biodegradable (refractory) organics are not removed by AWT processes, including the carbon absorption techniques. Some of these organics have been demonstrated to be harmful to health. We do not at this time have all the techniques to isolate, identify, measure, and partition these organics in terms of human health. Therefore, to emphasize our public health principle it is our responsibility to err on the side of caution in any consideration involving the possible introduction of such contaminants into a public drinking water supply.

Of paramount importance is the reliability of water treatment plant operations. For the sake of discussion, let us assume that the correct processes have been developed, validated and installed. What comfort does our knowledge regarding the operation of wastewater and water treatment plants give us pertaining to reliability? At present, very little.

The National Community Water Supply Study (1970) dispelled any reason for complacency in the findings that 36% of 2,600 individual tap water samples contained one or more constituents exceeding the 1962 Drinking Water Standards.

For adequate public health protection, it is clear that we need to accelerate our research and development to provide fail-safe operations, monitoring and control, particularly where drinking water is involved (all, of course, in addition to research for development and validation of processes which can do the job of providing renovated wastewater with no public health threat). A few of the operational principles and features which we foresee are:

- 1) Provision of standby units in case of unit breakdown most important for disinfection.
- 2) Provision of a "holding" basin between the wastewater treatment sector and the drinking water intake to allow for monitoring and bioassay prior to reuse.
- 3) Provision of an alternate means for disposal of the renovated wastewaters in case of long-term breakdown, and
- 4) Establishment of fail-safe monitoring and warning devices to maintain a continuous control on the system.

In summary I would like to cite the following: Before development of intimate personal contact uses of renovated wastewaters we need to:

1. Continue and expand studies on viruses to:
 - a. Develop improved viral detections and enumeration methodology.
 - b. Explore and define the basic properties of enteric viruses.
 - c. Provide knowledge on transmission of viruses through the aquatic environment.
 - d. Define the impact of viral disease on man through associated epidemiological studies.

- e. Develop technology for the positive removal and inactivation of viruses.
2. Investigate the potential for bacterial and other microorganism problems in reclamation systems.
3. Identify and define the potential health effect of organic and other chemicals, not removed by reclamation plants and subject to buildup, and develop techniques to identify and readily measure the concentrations of such chemicals.
4. Dispel the cloud which hangs over the whole subject of reliability for wastewater treatment plant operation. Reclamation plants for direct reuse must have fail-safe processes, backup facilities, alternate means for disposal, continuous monitoring and bio-assay and they must be operated in an atmosphere that demands reliability. State programs responsible for the operation of wastewater treatment plants will require upgrading. Pilot and field-scale testing will be required for the validation of processes and practices prior to their widespread use.
5. As a final point, use our common sense; renovated wastewater should not be used for the ultimate personal use, that of a drinking water supply until there is no other practical choice and, hopefully, when it needs to be used, the minimum research will have been completed and the use will be carefully operated and controlled. Meanwhile, in water-short areas, the renovation and reuse of wastewaters for industrial, limited irrigation and other low human-contact purposes should be investigated and advanced.

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OPEN FORUM

DR. COOPER (University of California, Berkeley): During the next hour we have scheduled an open forum which should be the interaction between the people who spoke this morning and the audience. As you see, some of the people who spoke this morning have left. We will conduct this open forum as long as it's reasonable to do so and then start the next session on general toxicology. I have a question. Do bacteria play their proper role in the vermiculite ecosystem model?

DR. METCALF (University of Illinois): That is a logical question and it is always asked. We are busy studying the microbial aspects of the system right now. We also have a number of programs where we are using soil. We have standardized on two types of soil that we are using in the ecosystem and comparing the effects in different soils and with and without vermiculite. As you would expect, there are great differences in the rate of retention of materials in the soil and perhaps some degradation, although the degradation patterns seem remarkably similar. We need to learn more about the microbial aspects of the system.

DR. SLONIM (Aerospace Medical Research Laboratory): Dr. Grenney, I noticed you emphasized that you used BOD measurements in your water re-cycling systems and you spoke of monitoring these systems at critical periods. Your slides show this to be a daily procedure. Did you use a more rapid method or just stick to BOD's for measuring oxygen demand? It seems to me that some of the chemical instrumental methods would have been much more adequate and would have given you a better assessment of the daily rate. Otherwise, you would have to wait 5 days in order to be able to tell what the BOD levels were.

DR. GRENNEY (Utah State University): We used the standard 5 day BOD and we did have to wait. We had 92 sampling stations on the Weaver Basin. At seven of those stations, we ran diurnal studies on BOD, DO, the various chemical species of nitrogen, phosphorus, suspended solids, pH, temperature, and flows. But we did use the standard bottle technique for BOD determinations.

DR. PETERING (University of Cincinnati): Dr. Mount, you brought out a very interesting concept of biological effects. On the other hand, it seemed to me that you were really talking about toxicity because you kept speaking of acute and chronic toxicity and I'm wondering whether you can clarify what you mean by biological effects. In mammalian toxicology we have come to look at effects on physiological function which do not necessarily produce overt toxicity, but that could be meaningful with respect to chronic disease incidence. With respect to aquatic organisms, what do you mean when you speak about biological effects?

DR. MOUNT (National Water Quality Laboratory): I think you have raised several good points. We really are unfortunate in our field of work to study physiological effects that are not obviously adverse in some other way as a measure of damage. My experience is that courts of law are not impressed by liver enlargement in large mouth bass. What they are impressed with is fewer bass in the fisherman's creel. That they seem to understand. Fish floating belly up is something they understand. Changes in physiologic function have been very disappointing in their utility in adversary proceedings. So my comments were really related to obvious adverse effects, like death, inhibition of growth, reproduction and egg fertility. These are effects that you can clearly establish are wrong. A point which you also alluded to was ignoring what happens after discharge of water. I did ignore that point and it's because the problem of dealing with unknown mixtures of variable composition is so immense that we would like to bury our head in the sand right now and pretend that it doesn't really happen. Maybe by the time we get our eyes above water, someone will have developed a maxi-ecosystem in which such studies can be conducted. I think this has to come. I really think that the behavior of chemicals, their deposition sinks as well as their breakdown products, is an area which must receive a great deal more attention even at the expense of toxicology studies. I think the 307A hearings which were held in February, March and April very clearly pointed this out. It was the unanimous opinion of EPA attorneys that the toxicology data was the strongest point we had in the whole hearings. If I put "X" pounds of a pollutant into a body of water, what will the concentration be today, tomorrow, and at some future time? Where will it go or how long will it remain there? Those were the questions that were left unanswered. If Congress and other legislative bodies insist on effluent limitations, you ultimately have to translate environmental concentrations to pounds per day out of a pipe. That's where information or data was lacking.

MR. WANDS (National Academy of Sciences): You made quite a point, Dr. Mount, of the comparison between the difficulties of aquatic toxicology versus mammalian toxicology. I wonder if aquatic toxicology has progressed in its selection of test organisms to the problem which mammalian toxicologists are now facing, that of an almost infinite variety of strains and hybrids available to select their species from for testing and thus the associated variation in susceptibility.

DR. MOUNT: I don't know whether we've progressed or digressed or what we've done. All I can do is tell you my thinking at the present time on how I see our problem. What we have to do is take a handful of animals and determine what's good for the United States. It seems to me if that's what we have to do then we certainly don't want an inbred strain of animals. What we need is a heterogeneous gene pool that has in it variability so that we measure some of that variability in the testing. To that end, I started a program when I was at Cincinnati in the early 1960's in which we intentionally

introduced new genes into our gene pool of fat-head minnows every year at the hatchery. We brought minnows in from Minnesota and other parts of the country for breeding. I do think work with test animals from one spawning adult female certainly does reduce variability but I'm not that sure we want precision at the expense of accuracy.

DR. CROCKER (University of California, Irvine): I was impressed with Dr. Metcalf's description of the system in which he was studying multiple biologic species. Have you had an occasion to use this type of system for sewage problems because it would seem to be the only way you could match the variety of biologic specimens that you have to protect.

DR. MOUNT: We haven't used it in the way in which Dr. Metcalf did. The use of micro ecosystems to predict effects is not well developed yet. I think Dr. Metcalf has primarily been looking at movement and breakdown of materials. The thing that was running through my mind as I looked at his diagrams is how can one convert that into a system where you could really look at adverse effects. The one obvious way is to scale it up to a much larger and perhaps dynamic system. In a sense that's really what we're doing at one of our new field stations. We have just opened a field station northwest of Minneapolis where we have eight channels that are about a fifth of a mile long. We have hydrodynamic conditions in these channels and we can really look at the fate of pollutants as well as effects because the system is large enough. This is probably the first effort in that direction, but such things are obviously immense and extremely expensive to operate. I'm not sure how much of that we can afford to do. If we're really going to talk about the interactions and competition among species, we've got to be able to test them before we can make any predictions.

DR. COOPER: Dr. Metcalf, do you agree with that?

DR. METCALF: I agree exactly with what Dr. Mount said. Our initial efforts were designed to prevent compounds from getting into the environment which were undesirable. We thought we had to have a way to prove it. The system's been, I think, very useful for that purpose. It certainly demands a considerable amount of sophistication to develop the kind of system Dr. Mount was describing. The biggest difficulty, of course, is being able to trace nonradiolabelled materials and I think mass spectrometry is now becoming sophisticated enough to do this. Dr. Mount has one of the best mass spectrometry labs and certainly one of the best mass spectrometers that I've seen. I think with people like that we can do just what you said.

DR. THOMAS (Aerospace Medical Research Laboratory): Dr. Spear, I suppose although you didn't mention it, or maybe I missed it, you do have a good history on these individuals as far as possible on their nonoccupational exposure in the garden and at home.

DR. SPEAR (University of California, Berkeley): No, I didn't mention it. In fact, the men we've used in both of our exposure studies so far are not professional pickers but are essentially students and staff at Berkeley. The reason that we used students is because they were confined for the 3 weeks of the experiment. We all lived together in dormitory accommodations and no one did anything or ate anything that we were not aware of. During the exposure sequence, we collected 24-hour urine samples, 5 days a week, for the analysis of the urinary metabolites. There is no way you can get professional pickers to live in that manner. We have great confidence that there were no other sources of exposure during this period.

DR. DREW (National Institute of Environmental Health Sciences): You didn't mention anything about particle size. Did you make any measure of particle size and did you correlate particle size with the concentration of the pesticides?

DR. SPEAR: We did some work on that last year, but only rudimentary results have been received. We used a Casella sampler mounted in the area where the men were picking. We took sufficient samples to be able to get oxon-thion ratios, a function of particle size on the four stages of the Casella sampler. Essentially the oxon-thion ratio increases with decreasing particle size, but not quite as dramatically as we had expected. As I recall, and as I say these data are quite preliminary, it changed by about a factor of 2 to 3 from the largest particles to the smallest ones that are fractionated in the Casella. That is the only size related chemical work we've done so far. We, of course, size the particles optically, but in order to collect enough to conduct a chemical analysis, we've used the Casella collection device.

MR. WANDS: Are they indeed respirable?

DR. SPEAR: Yes, generally speaking. I mentioned earlier that we measured dust particles in our studies on professional pickers at dust concentrations of 10, 20 and 40 mg/m³. The mass median diameter of those particles was on the order of 2 microns.

DR. HENDERSON (Olin Corporation): We've run into problems in getting preplacement physicals on contractors' people who are drawn from labor hiring halls. I'm wondering with the migrant worker population, is there a problem getting physician's certificates that these people could do the work with respirators?

DR. SPEAR: We had a physician there during the experiment and we gave the subjects physicals prior to and after the experiment. There was a physician in the field with the subjects observing them all the time. He took rather frequent pulse rate measurements during the day.

DR. CANTOR (Environmental Protection Agency): Dr. Cooper, I wonder if you could comment on the status of any epidemiological investigations that are taking place in the San Francisco area right now. It would seem to be an ideal area for that kind of study because of the variations in asbestos of various local water supplies.

DR. COOPER: I'm glad you mentioned that. It sounds like you read one of our proposals. We have, I think, a unique opportunity. We are having a very difficult time getting funded. And it turns out not only ourselves but also the State Health Department has put in a very similar proposal that didn't get funded either. One of the problems in associating water supply with disease or any other environmental phenomena to human disease is the reporting of the disease itself. Death is usually our best indicator because you can't die very well without being reported. So death as an indicator is pretty good. In the case of tumors, we have in the Bay Area one of the best tumor registries in the country. When we get down to the census tract data, we can compare social-economic groups. Piedmont is an upper class area as opposed to some areas in Marin County; Piedmont being on East Bay water, the other being on Marin water, one containing asbestos and the other not. So we have, I think, an ideal opportunity but there are a lot of problems. One of them is mobility of the population. It's pretty tough to prove a cause-effect relationship unless you deal with older people who have lived there 20 years or more since such a time period between exposure and disease has been reported among the exposed asbestos workers. It could be 20 or 30 years perhaps, so that the cause-effect relationship is difficult to establish. A bigger problem, but I think one that ought to be addressed, is how do you figure out what the average water quality has been over a 20-year period for these people who have drunk it. We got excited over some data the State Health Department had generated on the 4 large cities in Marin County comparing cancer death rates there to cancer death rates in the state at large and cancer of the colon, particularly in males. It was considerably higher in Marin County than you would expect from the rest of the state data. Of course, we got all excited about that. When we looked at morbidity data for the whole county, not just for those cities, the rate compares with the state as a whole. Morbidity-wise the number of cases didn't seem to be different, although that was for the whole county which uses a number of water supplies not just the ones we're talking about. Yes, we've considered an epidemiologic study but as far as I know, no one's doing one. I think agencies have not been anxious to fund such studies because of the lack of a standard method for analysis of asbestos and a lack of data on water levels of asbestos over the past 20 years. I don't know how we can go back and get that data, or what the asbestos level has been over that 20 years. There is a lot of valid criticism about such studies but I still think they ought to be done.

DR. CROCKER: Is it possible to get water supplies heavily contaminated with asbestos without there also being contamination in the air?

DR. COOPER: Do you mean contaminating the water supplies from the air?

DR. CROCKER: No. Duluth, Minnesota air has asbestos fibers in an airborne state as well as in the waterborne state. Obviously, any epidemiologic study is going to get a bit confused if you've got two potential sources, and certainly, being airborne does not mean that the asbestos fibers are only going to be deposited in the water.

DR. COOPER: We have a report almost ready to come out. We have been measuring asbestos concentrations in air around freeways and away from freeways in California. The air concentrations of asbestos are significant, of course, on the freeways and very low away from the freeways. The question is whether the asbestos in the air comes from the water or the asbestos in the water comes from the air. It is possible that water with high levels of asbestos may result in asbestos being aerosolized out of the water. I don't know what this means in terms of human health, but apparently we've been exposed to asbestos for a long time in California. It would be very nice to conduct a decent epidemiological study to look at these populations that we can isolate through census tract information and hopefully have some idea of where their water supply comes from.

DR. CROCKER: Are you taking air samples for asbestos analysis in the same areas as your water samples are collected?

DR. COOPER: We haven't but I think the State is doing that. We have not, mainly because we don't have any money. It costs us \$288 per sample so it's pretty expensive to do and it takes a long time. No one can just get the electron microscope and identify these fibers very well. It takes someone well trained in this area.

DR. STEMMER (University of Cincinnati): In these tumors that are allegedly developed from asbestos, do you find asbestos bodies?

DR. COOPER: My experience is no. I'm not an expert at tumorigenesis.

DR. STEMMER: How do you relate it then to asbestos?

DR. COOPER: These are Dr. Selikoff's data, that you are referring to?

DR. STEMMER: I am talking about those people that are exposed occupationally. You are looking at the general population, and I would like to know how you relate the development of colon cancer, for instance, to asbestos.

DR. COOPER: At this point, we have no data that relates them. I mean, we have no evidence that I know of that relates those cancers to asbestos. How do you actually confirm that the etiology of the pathology was asbestos? I don't know of anyone who can do that yet. There must be some cancer research people around that can answer that question better than I can.

DR. BUCHWALD (Environmental Protection Services): I'm going to develop this argument a little bit more this afternoon, but we're going through a little bit of a numbers game with asbestos fibers. One thing which ought to be rather important to toxicologists here is to look at mass relationships of the fibers. The great majority of fibers you find in water are less than 1 micron in length. In the data of Cunningham and Pontefract, which you quoted, they tried to quantitate the fibers they found on a mass basis and showed that approximately ten billion fibers were equivalent to 1 milligram. So looking at this on a quantitative basis, in comparing it to our usual toxicology terms of dosage, how much mass have we got when you're looking at one million fibers per liter?

DR. COOPER: Pontefract's number was, I believe, one microgram per million.

DR. BUCHWALD: No, one milligram of those asbestos fibers counted approximately ten billion in number.

DR. COOPER: The number I had was one microgram per million. I understand that it is difficult to get a correlation between weight and fiber count.

DR. BUCHWALD: The way they get a correlation between weight and fiber count for chrysotile asbestos is to separate the fibers into the fibrils (they take a known weight of chrysotile and ultrasonicate them to separate into individual fibrils), and then they obtain a relationship. But before we become too concerned about this from the toxicological basis, just look at the mass of asbestos you have when you're looking at one million fibers per liter, and relate this to dose, and certainly one can become very unconcerned about this.

DR. STEMMER: I come back to the asbestos fiber in water ... not that I'm very persistent, but I would like to make a comparison. We know in an epidemiological study that cigarette smoking increases the incidence of pulmonary carcinoma. There is no one who will state that in the smoke a particular compound is the carcinogen. Why do you come to the conclusion that the small amount of asbestos in the water is the carcinogen, when you also have a host of organic chemicals that could very well be carcinogens? Wouldn't it be much better to say that a certain water has a carcinogen in it, whereas another water does not, and not be so sure about the asbestos?

DR. COOPER: In defense of myself, I didn't draw the conclusion that asbestos in water causes cancer. There are inferences being drawn in many circles about occupational exposure data and that, because asbestos seems to be involved there, it's dangerous to be imbibed in the water supply. What we would like to do, now that we can tell whether asbestos is present or not, is epidemiologically to associate water supply with the incidence of disease. Frankly, I think the feeling among our group is that asbestos isn't very important. Most likely we won't be able to see anything, but we are interested in conducting a study. We're also interested in the opportunity to look at people who drink water from the Sierras, people who drink water from the local supplies, and other people, particularly in Contra Costa County, who drink all of their water from the Contra Costa canal. The latter has probably been through the intestines of the city of Sacramento and Stockton and a few other places, which would be sort of like a water reuse study. If there was any relationship between disease, in this case cancer, and water quality, you might find the same kind of relationship that you see with hard water and heart disease. But what the real cause of the correlation is, no one knows, because there is also a positive correlation between hard water usage and automobile accidents. Just because two factors are correlated doesn't mean that one causes the other. There's such a hullabaloo going on about the asbestos in water supplies that somebody ought to look at the data and either find that there does seem to be a relationship between water quality and this particular kind of disease or there is not. I was not pushing our area for study only because we have an excellent tumor registry that is broken down into census tracts and we do have this variety of water supplies. Dr. Buchwald, I wonder if you could comment on your experiences in Canada with asbestos.

DR. BUCHWALD: I am chagrined by the gross extrapolation from the occupational situation to drinking of water supplies and even asbestos ingested in fairly large quantities. As you probably know, Canada supplies about 35% of the world's asbestos in the form of chrysotile, the majority being mined and processed in Quebec. We obviously have occupational groups, particularly in the mining and milling industry, which are worthy of study and should produce a wealth of information from the epidemiologic point of view. The studies by McDonald and his co-workers at McGill University, Department of Epidemiology and Health, have been quite significant. The cancers which have been observed in Canada only relate to occupational groups which have extremely high exposure to the dust. Those occupational groups with moderate and lower exposures, in fact, don't show a very significant increase in the incidence of cancers, either respiratory or gastrointestinal. If one relates the dosage of asbestos a worker gets in the occupational situation to the possible dosage of a member of the public as a result of his water supply, there are orders of magnitude, many orders of magnitude, difference. Comparing the occupational situation to oral intake from food and drink is rather interesting. Work in Britain, particularly by Morga and co-workers at Hollowell, where they fed radioactive asbestos to rats, showed that 99.9% of the asbestos fibers are

passed directly through the gastrointestinal tract and not retained. On the other hand, following inhalation, a considerably higher proportion of the fibers are retained. Again, it's orders of magnitude in difference. Following inhalation, more than 10% of the fibers are retained in the lung, provided they are of respirable size. Thus, the majority of ingested fibers are excreted while those that are inhaled are likely to be retained in the lung. As far as the occupational situation is concerned, a great many of the asbestos fibers which are actually inhaled are impacted in the upper respiratory tract and eventually swallowed, so that a worker will inhale a lot of asbestos fibers and retain them. He will also swallow some and pass them through his GI tract. You would therefore expect, if there is going to be a gastrointestinal effect, that the workers would show a much higher response than members of the public. If doses are calculated, there are orders of magnitude in difference between occupational and public exposures from water supplies and other sources. Epidemiologic data for the heaviest exposure group in the Canadian industry is not to be confused with the U.S. experience of Selikoff and others. The increased incidence of respiratory cancers is perhaps three or four to one in the heavy exposure group, and the increased incidence of gastrointestinal cancer is only about two to one. Thus asbestos is not a potent carcinogen and this is why I drew attention this morning to the mass relationship. Generally, when we have a potent carcinogen in the environment, we're looking at parts per billion levels and a significant concentration of a carcinogen runs into parts per million levels in the environment. For chrysotile, looking at 10 million fibers per liter of water, is about 0.1 micrograms per liter or 0.1 parts per billion on the mass relationship. Is this a concentration giving rise to concern? We must be very careful in defining the health effects of asbestos. It is necessary to distinguish between the fibrinogenic effect in the lungs, the asbestosis, which is the most important occupational disease resulting from asbestos. Then there are the bronchogenic cancers, which must be distinguished from mesothelioma. Mesothelioma has received the greatest publicity because it is so highly malignant. In every case that's diagnosed, the result is fatal; there are no remissions and no useful treatments. It's rapid in its progress. From the time of diagnosis to death is a matter of three to eighteen months. It's a depressing picture and naturally the public becomes very concerned about this. On the other hand, how many cases of mesothelioma are there? In Canada, the annual incidence is about one per million of population. In the total population of Canada, twenty million, there are about 20 cases appearing each year. In relation to other diseases this is quite rare and we obviously can't spend a lot of resources probing into mesothelioma when there are causes of illness and death of much greater concern. Bronchogenic carcinoma is a completely separate entity from mesothelioma. As far as occupational exposure is concerned, lung cancers rarely appear in individuals who do not smoke cigarettes. The majority of the lung cancers resulting from asbestos occur in workers who also smoke. In addition to this, very few of the lung cancers in asbestos workers occur in individuals who don't already show some asbestosis; that is, the fibrogenic disease precedes the carcinoma. And in terms of prevention of the disease, if we apply

control measures which will essentially eliminate the asbestosis, we also eliminate the cancer.

DR. COOPER: In the Canadian experience, have they noticed any increase in intestinal cancer among asbestos workers?

DR. BUCHWALD: Only in the very high exposure group. Even then the increased incidence is quite small. I haven't got the data with me, but I think it's about two to one. This is a very small proportion of workers who were exposed for many years to very high concentrations of dust in their working environment. The quantity of asbestos which they ingested during their lifetime must have been on the order of many grams as compared to milligrams in terms of public exposure. There's a wealth of epidemiologic information available in those areas where the asbestos is mined. In the towns which surround the mining areas, there's considerable contamination, both of water supplies and of the air by asbestos, obviously. As the material is being mined great mounds of tailings appear. These tailings are washed into the streams by rainfall. It presents a very good situation for study and up to now nothing startling has appeared regarding any increased incidence of cancers in the exposed Quebec populations. As a result of this, we can't become overly concerned about the public exposure to asbestos. It isn't a priority situation. If we have to spend resources on research, it is unlikely to be on public exposure to asbestos. The occupational situation is quite different. There are some really significant exposures to asbestos and a significant incidence of associated disease; resources must obviously be spent on preventing exposure of the occupational group, but this bears little relationship to the public exposure.

DR. COOPER: As I pointed out, the tumor data in Marin County, which has the most asbestos in its water supply, is not different from the rest of the state. The death rates are a little different from the state at large for some reason, but the morbidity data that we have so far for the whole county are no different than the other five counties in the Bay area.

DR. BUCHWALD: The unfortunate thing is that the majority of publications, that deal with the finding of asbestos fibers in water supplies in the general public environment, always begin with talking about the health effects of asbestos in the occupational situation, whereas they don't have any evidence that the public is going to be affected. They leave you with that impression in mind, and immediately everybody thinks of doom from cancer as a result. The actual doom that is spelled out is mainly for those individuals who are occupationally exposed, as far as lung cancer is concerned anyway, who smoke because in this particular case, there is a multiplied effect. It is not merely additive. There's obviously some synergistic action between cigarette smoke and asbestos. An important inference from this is that lung tissue which is the specific target for cancer, and lung cancer is vastly different from the

tissue of the gastrointestinal tract. How on earth one can extrapolate from the lung situation to the gastrointestinal tract I fail to understand, but some of the physiologists here might tell us of some similarities.

MR. WANDS: I would like to make two comments that relate to what Dr. Buchwald has been saying. About 5 or 6 years ago, we were asked by the Public Health Service to make a survey of mesotheliomas in other organs besides the lung. This was obviously before the Duluth water problem became so popular. We were able to find something, I forget the exact number now, but less than 10 cases of mesothelioma which, of course, is pathopneumonic essentially for asbestos exposure, and 90% I'd say, let's assume we'd found 10, I think it was less than that, but of the ones we found, only one was outside of the gastrointestinal tract. And that one was reported to be a mesothelioma of the uterus, and it was the only case in which the exposure was described. Going back into the others, we could not identify the source of the presumed asbestos exposure for ingestion. But in this one case, a native living I believe in Transvaal or one of the South African nations, in the vicinity of some of the asbestos mines there and again exposed to the tailings which you spoke of that you have encountered also. She underwent a very spontaneous abortion and the local midwife or witch doctor, whoever else attended her, rinsed the uterine cavity with water downstream from one of these asbestos tailing deposits. About 2 or 3 years later she developed a massive tumor in the uterus which was initially diagnosed as mesothelioma. In looking into this a little bit, we checked with some of the other pathology people in the area and it turned out that the pathological diagnosis of mesothelioma of the uterus was in error. This was not a mesothelioma, so the upshot of this is, you've got to be sure that you know what your pathologist is doing when you start talking about some of these relatively rare tumors. And secondly, mesothelioma of the GI tract is a rare situation but does occur. There are a few cases on record.

DR. COOPER: Are there any other comments on the asbestos situation in water? I would like, if there are not, to talk a little bit more if we can, as a group, about water reuse in the United States. We're concerned with this matter in California. We haven't thought so much about asbestos but more about viruses and certainly about the organic chemicals that may be present and what the impact of these may be over the long pull. I would like to know how a toxicologist would approach the problem. In California, they are talking about getting a "blue ribbon panel" together to help the State decide how they should proceed in this problem of water reclamation. One of the questions that always comes up and is posed by the Health Departments is what I think Dr. Pringle said this morning. You can't allow anything bad in the water. The water must be safe. And the questions, of course, keep arising. What is safe? You can't demonstrate safety very well. What is an acceptable level? What is an acceptable way of going about looking at reclaimed water and making some judgement that it's not safe?

Would it be reasonable to use the same or a similar kind of philosophy as perhaps the Food and Drug people use, some "x" concentration that doesn't affect some test animal and therefore is safe? Does anyone have any comment on this point? Let's assume that we could concentrate organics in water down to where we could get a kilogram of these organic compounds out of water. What kind of levels would be acceptable? A thousand times what an animal would normally be exposed to? I'm very anxious about this because we have to be able to talk about risk in setting standards.

DR. PETERING (University of Cincinnati): Aren't we talking about things that are already being done when industrial concerns are dumping materials into the water? In a sense, you are reusing that in the natural system that you are already working with. In the Ohio River, there are at least 12 or more carcinogens that have been identified in the water at levels which are variable but from which nobody knows what effect will be produced over a very great length of time. It seems to me that in this discussion of reuse, one ought to go back and take a look at what's happening in the natural systems where many organic pollutants are present and nobody's worrying about them. I think we ought to put these ideas together in context to try to understand what we already have and make this to some extent an internal picture of what organics do in the water systems. Those systems that we already have as well as reuse systems. It seems to me that we ought to consider what is already happening with the sewage discharge that we all lightly pass over by saying that, "Well, if you take a look at the Merck Index, it's all there." That doesn't say anything to anybody and it really smears the whole picture. It seems to me that the reuse of the natural systems, natural waters, ought to be looked at, too, because we really are reusing the water over and over. The turnover time in the large pool of water in the Ohio Valley is probably many years, but certainly not a matter of hours or days.

DR. COOPER: In talking with health officials, it appears that if a pollutant enters the Ohio River and then is passed to an individual, it's all right. However, if you intentionally take waste water and upgrade it and then put it into the ground or directly into a water system, that is a different system now, and it has to be proven safe. I'm still wondering how we would make some judgement as to what's an acceptable pollutant level.

DR. CAVENDER (Becton-Dickinson): I would like to add another dimension to this. We haven't talked at all about heat pollution from cooling systems of electricity, generating plants and so forth, or turbidity perhaps as mentioned in stratification of lakes. It seems to me that if we want to standardize on a single policy for water that we're cutting our own throats. That, if indeed, we are going to require an electrical generating plant to have an ambient temperature like the natural waters of the state, then we're forcing the price of electricity upward. We're forcing the system where we intend to reuse the water to be less polluted than natural water is downstream from city "A" to city "B". We're forcing ourselves in a very expensive direction. I'd like to see, perhaps, many standards rather than a single standard generated and perhaps comments from other people.

DR. BUCHWALD: Just the mention of several pollutants from generating stations brings to mind a situation in our province of Alberta. There is a 2 megawatt coal fired power station close to a very large lake, and we have the problem of thermal pollution there. Yes, it may be desirable to have the water a little warmer but it creates severe problems with plant growth in the lake. The whole area around where the warm front goes into the lake has an excessive growth of plant material and it's beginning to block up that portion of the lake. We are looking towards this waste heat as possibly a usable resource. There's experimental work in progress at the moment in terms of heating greenhouses, very useful in adding to the short growing season in that northern climate. A great deal of work is being done now in trying to utilize some of these wasted resources. However, I did particularly want to say a little more about recycled water problems. It was just yesterday morning that I visited the Ontario Research Foundation at Sheridan Park near Toronto, where they have a demonstration project of a waste water process. This project is supported by the Central Mortgage and Housing Corporation. In Canada, there is a very real problem about the supply of potable water to residences in northern communities which are frostbound for many months. The Ontario Research Foundation has great difficulty in getting the health authorities to define what is an acceptable water. They can come up with all sorts of limits for certain individual chemicals but the authorities will not approve the use of recycled water. There is a gut feeling somewhere about the problems of viruses, the problems of certain undetected organic chemicals, and this was very eruditely explained to us by the last speaker this morning. I think that these are very real problems and as toxicologists you're quite right in saying we must try and develop some kind of methodology and some techniques for testing this very valuable resource, namely water. Last week I had the opportunity of speaking to Professor Sontheimer from the University of Karlsruhe, Germany who is working on very similar problems concerning the river Rhine. In North America, we don't have any problem waters quite as bad as the river Rhine. He claims that they have a perfectly usable system based first of all on primary and secondary treatment of sewage followed by filtering through activated carbon. He claimed that they can get rid of most of the objectionable organic matter like the halogenated compounds and they finish up with an ozonation which (they claim) will get rid of most of the pathogenic factors. Apparently in Germany they also object to chlorination for the reason, possibly, that it adds to the problem of organic halogen compounds in the water, and they are using a system of ozonation with a great deal of success. There are workable systems. It does appear that the water is very good after treatment. At the Ontario Research Foundation, they showed me several goldfish in the outfall from treated sewage where they had been swimming about for quite a long time. The researchers thus are convinced that this water could be introduced back into the public water supplies but it's a question of health approval. I would say that a fish is a very good indicator for chemicals in the water supply. They are exquisitely sensitive to many chemicals, much more so than man and I want to

emphasize something that was pointed out this morning, namely their avoidance of water which is contaminated. The questions that fish don't answer are related to organisms which are pathogenic to the human.

DR. STEMMER: I think from the practical point the problem is not so much to identify the materials that are in the water, and I am talking about the chemicals, because that would be a very large task. Would it not be better to develop a test system which will use the water itself in the test? I have a question for Dr. Crocker. Could tissue culture or organ culture be used for water assay, especially if one could concentrate some of the pollutants?

DR. CROCKER: I don't think I really deserve the honor of speaking for the in vitro systems as they may be used. They have been used. The Department of Microbiology at the University of Texas in Houston has been doing water filtration research for quite a long time, concentrating sewage or contaminated water in search of enteric viruses, and the search depends upon growing the viruses on cell cultures. This is the kind of pathogen I think we were talking about where identification of the virus by this means is quite readily done, and that same kind of project is being undertaken at UC, Irvine in cooperation with the local Department of Public Health, Water Sanitation Division. In general, the findings I have been made aware of have been that the amounts of viral contamination in raw water and also in processed water are reasonably parallel with E. coli counts and as E. coli counts go down in the course of water purification so do viral counts. The only unsettled question is that there is always a residue of virus in treated water supplies and whether that residual amount is important is not known. It may be that Dr. Robin who is here and is interested in in vitro systems would comment further on this point.

DR. COOPER: Dr. Crocker, I think the question was in regard to unknown chemicals. Is it a good system for chemical assay?

DR. CROCKER: I think there is much less known about the possible applications of in vitro systems for that purpose. At that point you would be not much better off, I'm afraid, than if you were to simply analyze a concentrate, so I can't speak from any practical experience. Perhaps you should answer your own question.

DR. STEMMER: This morning the question was raised, "Why does one analyze for certain chemical compounds when one does not know anything about the interaction of these chemicals in the water?" I think this is important and if one could use the water in tissue culture or cell culture one would have the combined effect. You have done work in this culture system in regard to carcinogens. Is there not a possibility to do the same in regard to toxicological evaluations?

MR. WANDS: I don't like to keep monopolizing the discussion here but many of the group, I'm sure, are aware of the fact that NASA has pioneered in

the recycling of water. Particularly in developing systems for providing potable water on board their orbiting laboratories and in the next five years to have these operational. At that time, they plan a long enough space flight with a large enough crew that it will no longer be feasible to carry their drinking water on board with them as we have been doing for the moon shots, for example. It will be necessary then to recycle the water. About two years ago, a committee of the National Academy of Sciences did give NASA some guidelines on both the bacteriological quality of their drinking water as well as the chemical quality of this and they went further than the 1962 Public Health Service drinking water standards which were somewhat limited in the number of chemicals with which they dealt. I don't remember any of the details at this moment of the standards which were set for drinking water. I do recall, though, that the group finally resolved one very difficult issue and that was that they would have two kinds of water on board, each with its own standard, one for potable water and for wash water for personal sanitation and showering. There will be showers in space labs. The other question I wanted to raise is about the business of recycling water. Dr. Buchwald mentioned that it's very common practice in Europe to use ozone for water purification rather than chlorine as we are doing in this country. It also, of course, eliminates problems with mercury contamination because we don't have to use the mercury cell to generate the chlorine for ozone. There are today about a thousand plants in operation in the world using ozone. We expect within the next ten years, there will perhaps be 10,000 ozone sewage treatment plants and most of those are likely to be in the United States because our supplies of chlorine are becoming more and more critical, and they can demand a better price for chlorine for use in chemical synthesis rather than for sewage treatment. Now, that situation may change as we get more and more stringent about chlorinated hydrocarbons in our living systems. If we set stringent limitations on polyvinylchloride, for example, which today takes about 25% of the U.S. production of chlorine, we may find that that prediction of ten years will change. I would also like to ask if anyone in the audience has hard data on the differential susceptibility of these pathogenic organisms, particularly of virus to ozone versus chlorine. It's been suggested that these viruses do seem to be a little more susceptible to ozonolysis, but I don't really know myself. I'd appreciate hearing some hard data.

DR. COOPER: I don't think there is any hard data. Otis Spraul from the University of Maine has generated some recently, but I gather the problem is the same problem we have in evaluating chlorination; each place does it differently and it's very difficult to compare one to the other. You must have very standardized methods. So I don't think there is any good data on ozone treatment of water yet. I understand, however, that in the European water supply where they use ozone, they still use chlorine as well to maintain a residual in the system which is one of the drawbacks of ozone treatment; to maintain water quality, there must be some chlorine residual in the distribution system.

DR. ROBIN (Stanford University): I don't have any hard data on anything but there was a triple play, Stemmer to Crocker to Robin, and I thought that I would respond. The question was, "Is it possible to use cellular systems to screen for biological effects of a wide variety of toxic agents?" I hope so because I think that's what I'm supposed to speak about on Thursday. More importantly, it seems to me that the only hope would be based on systematic studies or analyses in in vitro and cellular systems. Because of the sheer number of newly detected or developed toxic agents, isolated nonsystematic observations on a wide variety of agents become not only impractical but cannot keep pace with the rate of production of these problems. Thus, a systematic approach in which one can look into a wide variety of agents in a specialized and clear cut fashion would be highly desirable. With such in vitro systems, there is the advantage that it isn't necessary to isolate the precise chemicals involved. These testing systems could occasionally be used in a screening sense so that something as nondescript as recycled water could be tested.

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SESSION II

GENERAL TOXICOLOGY

Chairman

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COMMENTS ON A PLASTICS INDUSTRY NEUROTOXICITY
IN RELATIONSHIP TO METHYLBUTYL KETONE

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Lynn B. Hetland, Ph.D.
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INTRODUCTION

Recently a number of workers involved in a local plastics coating manufacturing plant were afflicted with peripheral neuropathies of varying degrees of severity. The clinical results of electrodiagnostic (EMG) screening of 1157 workers from this plant showed 965 had no abnormalities; 72 within normal limits; 24 with no definite abnormalities; 28 with suspected abnormalities; and 68 with definite signs, symptoms and electrodiagnostic findings of peripheral neuropathy (Mendell, 1974; Allen, 1974; Billmaier, 1974). A thorough epidemiological study revealed that the recent introduction of methylbutyl ketone (MBK) as a solvent in the dye and color printing processes might be linked to the neuropathy. This was suspected especially since plastics industries throughout the country had reported no known illness of this sort and processes were similar except that no other plant used methylbutyl ketone (Billmaier, 1974). It was inferred that methylbutyl ketone must be related to, or indeed the cause of, the peripheral neuropathy.

Analysis of air samples in the print shop area showed variable but consistently high ketone concentrations (methylethyl ketone, approximately 10-40 ppm). In addition to ketones, low levels of hexane, diacetyl, aromatic hydrocarbons and traces of other solvents were detected (Billmaier, 1974).

A search of the toxicology literature gave only a paucity of data available on MBK toxicity and essentially no studies on the effects of chronic exposure (Browning, 1965).

In response to the nature and urgency of this industrial toxicity and in view of the unknown agent(s) involved; a cooperative venture involving dozens of investigators at The Ohio State University emerged in an attempt to determine the cause of this neuropathy. This report describes our approach to the evaluation of methylethyl ketone and methylbutyl ketone as possible causative agents of the peripheral neuropathy.

METHODS

Toxicology Testing

Our initial efforts involved an examination of workers blood, urine, hair and nails for any contents which might lead to the identification of any known or suspected neurotoxin. A drastic limitation of this approach was that these biological samples were obtained from workers that had been away from the work environment for 3-6 weeks or more. Other samples were obtained from workers on the job only after MBK had been removed from the process. In any case, efforts were made to determine blood and urinary heavy metals, thiocyanates, ketones and ketone metabolites, total urinary glucuronides, thiamine, serum and RBC cholinesterases and plasma CPK. Analysis of heavy metals, lead, zinc, cadmium and thallium were done by atomic absorption spectrometry utilizing carbon rod atomization; mercury by flameless AA; arsenic by colorimetry. Cholinesterases were determined by the isotope method of Siakotos (1969). Other assays were performed according to currently acceptable standard methods.

Exposure of Animals

The purpose of these studies was to establish an animal model of peripheral neuropathy in order to demonstrate the etiologic relationship of a volatile solvent to this disease. We felt that the inhalational route was the most likely manner in which workers were exposed. We, therefore, utilized the inhalational route, exposing animals in a modified germ-free apparatus fitted for flow-through concentrations of vapors carried via a continuous air flow. Chambers were monitored to maintain O₂ at 21%, CO₂ at 0.1-0.2%, relative humidity at 30-50%, temperature 24 C. Concentrations of ketone vapors were determined by GLC and agreed with calculated volumes of liquid required to achieve the appropriate levels.

In addition, food and water were available in the chamber, consumption of food and water and body weight were recorded throughout the studies. Exposure to ketone vapors was considered at tolerable levels only if there was no diminution in food and water intake. If these were decreased, then ketone concentrations were lowered. Pair fed controls were subjected to similar conditions in the absence of the ketone vapors.

It was not readily predictable whether or not a peripheral neuropathy would be produced by ketone vapors or even occur in an animal model. In the preliminary studies we used several species which included chickens, mice, rats and cats. We found that all species except the mouse exhibited a typical clinical peripheral neuropathy. The chicken was the most sensitive species showing severe neuromuscular impairment at 100 ppm MBK after three weeks of continuous exposure. Rats were more manageable and motor nerve preparations were examined for ultrastructural alterations after solvent exposure. Cats exhibited peripheral neuropathies and were especially suitable for electrodiagnostic evaluations of neuromuscular function.

In all systems, exposure was continuous, 24 hours a day, seven days a week, but with the following interruptions: a 15 minute break each day for all chambers for measuring and replenishing feed and water; a 24 hour break once a week for the cats only, when they were removed for electromyogram studies. Also, at one to two week intervals, chickens, rats and mice were removed from the chambers for 30-60 minute intervals for obtaining body weights and blood samples.

Electromyographic Studies

Electromyographic studies and ulnar nerve conduction measurements were carried out on all exposed cats and their controls on a weekly basis according to methods described by Chrisman (1972).

In some exposure experiments, cats that exhibited early and marked neuropathies were removed from MBK or MEK-MBK vapors and were handled as controls for observation for recovery processes.

Histology

The sciatic nerves of animals exposed to MBK or MEK-MBK and their appropriate controls were fixed in situ with 3% glutaraldehyde in 0.1M phosphate buffer pH 7.5. The nerves were postfixated with 1% osmium tetroxide and embedded in Spurr low-viscosity media. Sections were studied by light microscopy. Thin sections of portions of the nerve were viewed with a Hitachi Hu 12 electron microscope. Other portions of the nerve were prepared for nerve fiber teasing (Dyck, 1968).

RESULTS AND DISCUSSION

Toxicology Testing

The results of heavy metal analyses in urine and blood of the workers are summarized in Table 1.

TABLE 1. METAL ANALYSIS

<u>Metal</u>	<u>Results</u>	<u>Assay Sensitivity (ppb)</u>
Blood (91 workers)		
Lead	within normal limits	5.0
Zinc	within normal limits	5.0
Mercury	within normal limits	1.0
Urine (91 workers)		
Arsenic	within normal limits	10.0
Cadmium	within normal limits	0.1
Mercury	within normal limits	1.0
Thallium	within normal limits	1.0
Zinc	within normal limits	5.0

All values were found to be within normal limits, or not detectable. Analyses for other nonmetallic agents (Table 2) were also within normal limits, the few elevated urinary thiocyanates were not considered significant and not pursued.

TABLE 2. NONMETALLIC AGENTS

<u>Number of Samples</u>	<u>Test</u>	<u>Results</u>
12	Acetone bodies (blood)	within normal limits
120	Acetone bodies (urine)	within normal limits
99	Halogenated Hydrocarbons (urine)	within normal limits
85	Thiocyanate (urine)	six elevated values

Additional analyses were carried out on the technical grade solvents MBK, MEK and the recovered recycled MEK-MBK. Gas chromatographic analysis using an alkaline flame ionization detector did not indicate any phosphorus containing compounds (e.g., TOCP and other organophosphates). Infrared

analysis of recycled solvent after fractional distillation failed to demonstrate any nitrile containing contaminant. In the search for any possible contaminants occurring in the workers environment, we were limited to the study of unused solvents MBK and MEK exclusively. Unfortunately, we could not obtain any material actually used in the printing process which would have included a mixture of solvents with various components of the colors, dyes, resins, adhesives, plasticizers, detergents, color stabilizers, antimicrobials and fire retardants. The advantage of examining the solvent mixture would be to include the possibility not only that one of the process chemicals itself was potentially toxic, but that a toxic substance might be formed by reaction between chemicals under the temperature, humidity and conditions of the printing processes. Also, MBK was no longer used in the plant and the crude mixture would lack components (if any) extractable or soluble in MBK.

Cholinesterase determinations of about 100 workers who were judged to have subclinical manifestations of neuropathy showed a significant elevation of serum butyryl cholinesterase and an equally significant fall in erythrocyte acetyl cholinesterase (Table 3, Group D).

In employees judged no longer EMG-positive and in ten employees not directly involved in the print shop operation, there was no significant difference in their serum and erythrocyte cholinesterase levels and all appeared to be within normal range (Table 3, Group A vs B). There is some indication that in at least a few cases where symptoms developed later, these individuals had unusually high plasma butyryl cholinesterase and low erythrocyte cholinesterase.

TABLE 3. CHOLINESTERASE ACTIVITY

	<u>RBC</u> <u>μmoles/ mg/ hr</u>	<u>SERUM</u> <u>mmoles/ ml/ hr</u>
A. Normals (workers) N = 10	1.74 ± 0.57	0.45 ± 0.15
B. Samples (print shop workers) N = 10	1.42 ± 0.48 (a) p > 0.5 (b) 0.2 > p > 0.1	0.37 ± 0.13 (a) p > 0.5 (b) 0.4 > p > 0.2
C. Normals (hospital patients with normal liver function) N = 14	2.03 ± 0.14	0.25 ± 0.02
D. Samples (workers -- initial) N = 96	1.37 ± 0.06 (c) p < 0.001 (d) 0.2 < p > 0.1	0.64 ± 0.02 (c) p < 0.001 (d) 0.05 > p > 0.02

Cholinesterase values represent the mean ± SEM
Significance levels, p values, by Student's Test

- (a) Comparison was made between groups A and B
- (b) Comparison was made between groups B and C
- (c) Comparison was made between groups C and D
- (d) Comparison was made between groups A and D

The influence of chronic exposure of mice, rats, and chickens to MEK on cholinesterase activity is presented in Table 4. It is important to note that there is in all cases a statistically significant elevation in plasma cholinesterase levels following chronic exposure. In some cases, MEK produced greater increases than MBK (data not shown) but this may be due in part at least to the much greater CT (concentration x time) experience with MEK. The rodents exhibit a significant decrease in erythrocyte cholinesterase which is not shared by the chicken. This may reflect the difference between avian erythrocytes which are nucleated, and hence capable of protein resynthesis, and rodent erythrocytes which are nonnucleated.

Determinations of serum CPK in workers as well as animals exposed to solvent vapors were in the normal range.

TABLE 4. EFFECT OF MEK EXPOSURE ON CHOLINESTERASE ACTIVITY^a

	<u>Control</u>	<u>MEK</u>	<u>CT Value*</u>
<u>Mouse</u>			
RBC	0.44 p = 0.02** p = 0.3	0.11 0.24	125 43,200
Serum	1.6 p < 0.001 p = 0.003	4.1 2.5	125 43,200
<u>Rat</u>			
RBC	0.40 p = 0.02 p = 0.03	0.14 0.19	125 43,200
Serum	0.15 p = 0.008 p < 0.001	0.30 0.27	125 43,200
<u>Chicken</u>			
RBC	0.013 p = 0.008	0.025	32,000
Serum	0.57 p < 0.001	1.3	32,000

^aRBC acetylcholinesterase activity expressed as $\mu\text{moles}/\text{mg}$ protein/hr.

*CT Value = Concentration (ppm) x time (days).

**p < 0.01 is statistically significant.

Exposure of Animals

After preliminary range finding experiments with solvents and species, the following exposure pattern was employed.

In separate m³ chambers, animals were exposed to vapors of MBK, MEK or a mixture of MEK-MBK vapors (10:1). In one chamber four cats, four rats, and five mice were exposed to MBK starting at 75 ppm and doubling at approximately weekly intervals until 600 ppm was reached. Another similar chamber exposed animals to a combination of MEK and MBK 10:1, starting at 500:50 ppm of each, doubling these levels to a maximum of 2000:200 by the third week. Another chamber with chickens alone had initial levels of 50 ppm MBK increasing to 200 ppm by the third week. In each case the peak level was to be maintained for at least six weeks, providing severe adverse reactions did not occur in the animals. Food and water intakes and/or body weights falling more than 10-25% below controls would be taken as a general toxic response likely to confuse the development of peripheral neuropathy, and would necessitate a reduction in solvent concentration.

Choice of solvent concentration and continuous exposure was dictated in part by the desire to obtain a rapid response but was modified by the appreciation that overwhelming toxicity would likely obliterate the more subtle aspects such as nerve conduction and EMG changes and possibly even gross signs of paralysis. Therefore, a relatively low initial level and a stepwise increase in solvent concentration was adopted, with continuous observation of animal performance along the way. Peak levels to be reached were established in preliminary trials in which rats and mice would tolerate 600 ppm MBK; cats might, and chickens would not. Also preliminary trials showed that 2000 ppm MEK was tolerable for rats and mice. It seemed prudent to increase chamber concentrations in a cautious stepwise manner. The ratio 10:1 for MEK:MBK was suggested as being representative of the working conditions in the plant.

The chronology of effects observed by continuous exposure of 4 species of animals to MBK vapors is summarized in Figure 1. The data indicate that chickens were the most sensitive species. MBK (200 ppm) caused some decrease in food and water intake in the first 3-5 weeks; the sixth week MBK was reduced to 100 ppm. In the fifth week, chickens showed signs of leg weakness, manifested initially by reduced activity and unsteady gait, soon thereafter (1-2 days) the chickens could not stand on their legs at all. From week 5-12, chickens were sacrificed for histology as soon as they exhibited the progression of mild to severe symptoms. The restricted-fed chickens (controls) had similar weight losses but no leg weakness.

Cats, rats and mice tolerated higher levels of MBK than chickens but, as seen in Figure 1, after several days at 600 ppm, a depression in food and water intake prompted a reduction to 500 then 400 ppm MBK. The first sign of neuropathy, positive waves in electromyography (EMG), also occurred

during 600 ppm exposure. Chickens developed severe paralysis in a minimum of 5 weeks in MBK 100-200 ppm, whereas cats showed leg weakness in this same time interval at 400-500 ppm MBK. Rats were more resistant than cats to MBK, requiring exposures of about 11-12 weeks at 400 ppm for the first signs of leg weakness to appear. Mice were exposed to 400 ppm MBK for 9 weeks and did not show any evidence of leg weakness. Mice gained weight comparable to controls.

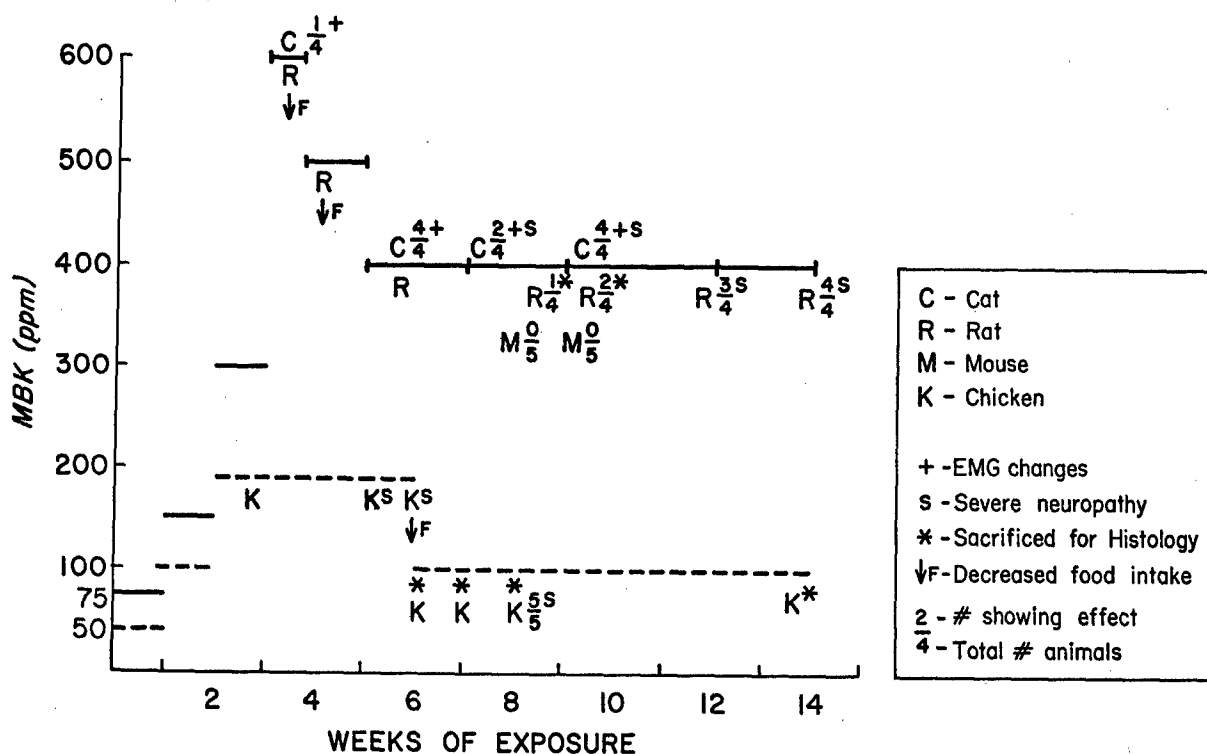


Figure 1. Effect of MBK exposure on various species.

Other experiments were conducted with animals exposed to vapors of MEK alone, or mixtures of MEK-MBK. The results are summarized in Table 5 depicting the solvent concentrations, time to occurrence of paralysis for each species, and the variation in response. Noteworthy is the absence of any paralysis in the mouse with all solvents tested. Also, exposures to MEK alone did not cause paralysis in any of the animals (exposure was 7-9 weeks, 1500 ppm).

TABLE 5. TIME TO OCCURRENCE OF CLINICAL PARALYSIS

	<u>Chicken</u>	<u>Cat</u>	<u>Rat</u>	<u>Mice</u>
MBK (ppm)	200 4 weeks	400 5-8 weeks	400 12 weeks	400 none
MEK (ppm)	1500 none	1500 none	1500 none	1500 none
MEK-MBK (ppm)	1500-150 4 weeks	1500-150 5-8 weeks	1500-150 6 weeks	1500-150 none

Interaction Between MEK and MBK

In rats exposed to a mixture of vapors of MEK-MBK, 1500:150 ppm, paralytic signs were observed in about 6 weeks; whereas in 400 ppm MBK alone 11 weeks elapsed before leg weakness appeared. Cats were somewhat wobbly in MEK-MBK 1500:150 ppm, as if partly anesthetized. While this unsteadiness did not appear equivalent to the leg weakness and to paralysis seen in MBK alone, it may be difficult to separate the two conditions in their earlier phases. Food intake was also more depressed in the MEK-MBK combination than with MBK alone. In addition, the MEK-MBK cats showed a prolonged duration of sleep after pentobarbital anesthesia (given for the electrodiagnostic tests). Mice were equally little affected by either MBK alone or MEK plus MBK. The data obtained from rats and cats exposed to MEK-MBK suggest that MBK is more toxic in combination with MEK.

Electromyographic Studies

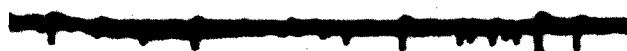
Each cat used in these experiments was tested by electromyography at weekly intervals prior to and during exposure to the solvent vapors. Recording of the electrical activity of the muscle at rest and during insertion of the electrode was obtained in anesthetized cats (pentobarbital 30 mg/kg). Typical electrodiagnostic data is presented in Figure 2. The top row tracings show positive waves which are the first sign of muscle irritability; these were seen as early as 4-6 weeks in all the cats exposed to MBK (see Figure 1). Fibrillation potentials along with positive waves (Row 2) indicate severe disease of the motor unit.

After 7-9 weeks, all the cats showed ulnar nerve conduction velocities slowed to about 50% of their 115 m/sec control values (Row 3 vs 4). The EMG findings occurred in all muscles tested, with the more marked changes noted distally. Cats exposed to both MEK-MBK exhibited electromyographic changes similar to those observed with MBK.



Figure 2. Electromyography patterns after MBK exposure.

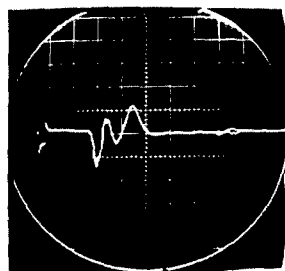
Top Row: Train of positive waves in the anterior tibialis muscle after 6 weeks of exposure to MBK in the cat.



2nd Row: Fibrillation potentials in the triceps after 9 weeks of MBK exposure in the cat.



3rd Row: Prolonged latency between stimulus and recorded muscle action potential from ulnar nerve stimulation (wrist to food pad on the left and elbow to food pad on the right).



4th Row: Control recording showing stimulus artifact and recorded foot pad muscle action potential from stimulation of ulnar nerve (wrist to food pad on the left and elbow to foot pad on the right).

Histology

During exposure to MBK or MEK-MBK, some rats, cats, and chickens exhibiting neuropathies of varying severity were sacrificed (see Figure 1) for histologic studies. Exposure to MBK or MEK-MBK, but not MEK alone, caused similar pathological changes in nerve preparations of all three species. A composite of results of the histologic examination after MBK is shown in Figure 3. The salient findings were: axonal swelling, oftentimes para Nodal (Figure 3a, 3b); denudation of myelin and thinning of myelin (3c); a greatly increased number of neurofilaments with fewer microtubules (3c). None of these histologic alterations occurred after MEK alone.



Figure 3. Effects of MBK on rat sciatic nerve preparations.

- a Teased nerve fiber showing areas of axonal swelling (arrows). Node of Ranvier seen at the arrowhead. X 100
- b Thick section of epoxy resin embedded nerve showing swollen axon and denudation of myelin (arrow). The normal size of axon can be seen at Node of Ranvier (arrowhead). X 500
- c Electron micrograph through area of axonal swelling showing thinning of myelin sheath (M) and decreased number of microtubules (MT) compared to neurofilaments (NF) which are increased. X 43,200

Comment

This study demonstrated that the continuous exposure of animals to MBK can produce leg weakness which progresses to a paralytic neuropathy in chickens, cats and rats, but not mice. Many clinical features of the peripheral neuropathy in the workers were also demonstrable in the animals, for example, muscle weakness, alterations in electromyogram, RBC and serum cholinesterase changes. The histologic changes in animals remain unknown for man since no nerve biopsies were obtained.

The results obtained do not necessarily prove the MBK was the causative agent in the human neuropathies. It is not inconceivable, and indeed, quite possible, that other substances initially present or formed in the process and extractable by MBK are the primary toxic agents. What our results demonstrate quite clearly is that MBK under the conditions described can produce a condition similar in many respects to the human neuropathy.

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CARDIOPHYSIOLOGICAL STUDIES WITH STRESSED ANIMALS

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INTRODUCTION

Toxicological investigations have, for the most part, used healthy animals or men as test subjects. Particularly, in the case of human studies, only the healthiest individuals are subjected to experimental protocols. As an example, only about 40% of the applicants (all supposedly healthy) are accepted into the human studies program at Marquette. The case for animal research is not quite so clear, but again, most of the studies are conducted on healthy subjects.

The real world, unfortunately, is such that all segments of the population -- for example, the very young and the very old, the healthy and the sick, the well fed and the hungry -- are exposed to environmental pollutants. Part of our task as environmental toxicologists is to identify subgroups of the population that may be particularly susceptible to a pollutant or to a combination of pollutants. The above mentioned factors -- age, general health, nutritional state, hereditary anomalies as well as many others -- may all play a role in the susceptibility or sensitivity to environmental toxins. Thus, the use of stressed animals in toxicological research may be emphasized more and more in the future.

Stressed subjects have only rarely been used in cardiophysiological studies (with the exception of studies investigating the effects of exercise). Anderson et al. (1973) have recently reported on the effects of low level carbon monoxide exposure on the onset and duration of angina pectoris in humans. That group is also investigating the effects of carbon monoxide on the severity of induced myocardial infarcts in both pigs and dogs.

In our laboratory we have been investigating the cardiovascular effects of various haloalkane propellant gases with closed chest rabbits as the test

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species. In one series of studies we have lowered the oxygen concentrations thus making the animals hypoxic -- a stress which has been postulated to be the sole cause of some results reported previously. We have then investigated the effects of trichlorofluoromethane (F-11) on these hypoxic rabbits.

In addition we recently became aware of a strain of hamster that develops heart failure as a result of an inherited defect in the cardiac muscle (Gertz, 1972). We have tested the effects of acute exposures to F-11 on this species of hamster, which may represent a stressed or susceptible model. This paper summarizes our results with these two variations of stressed animals.

METHODS, RESULTS, AND DISCUSSION

The biochemical and genetic character of the muscle defect in the cardiomyopathic hamster is unknown, but in the BIO 82.62 strain of Syrian hamster (available from Trenton Experimental Laboratory Animal Co., Bar Harbor, Maine), the disease follows a predictable pathological and physiological course. The disease separates into four histological and clinical phases: pre-necrotic, necrotic or myolytic, hypertrophic and terminal. During the first stage no clinical, gross, or histological pathological changes are notable. In the second phase, the maximal response consists of focal myolysis and necrosis and is only noticeable on histological examination. Healing begins at about 60 days of age and is usually complete by 90 days, but there is still a low incidence of both lesions. In the third or hypertrophic phase, clinical signs are still absent but animals killed at this time have secondary calcification of the myocardial fibers and the heart begins to dilate. During the fourth phase, the clinical signs of heart failure appear. The animals appear cyanotic, accumulate fluid, and die several days later. The particular strain of hamster used in this study was in frank congestive heart failure at the age of 240 days and at that time weighed an average of 24 gm more than 180-day-old hamsters. The old CMP hamsters appeared edematous and on necropsy had dilated hearts.

We initially compared the acute inhalation toxicity of F-11 on control versus cardiomyopathic hamsters. Three groups of 16 hamsters, each group divided into four subgroups of four animals each (150- and 240-day-old myopathic hamsters and 150- and 240-day-old random-bred hamsters) were exposed for 4 hours to either room air, 7.5% nitrogen-room air or 7.5% F-11-room air mixtures on the same day. Myopathic and random-bred hamsters of a given age were stratified by weight and then assigned to one of the three exposure groups at random. A fourth group of 16 hamsters was exposed to 2% F-11 in room air on a separate day, and another group of four 240-day-old random-bred hamsters was exposed to 10% F-11-room air. These results are shown in Table 1.

TABLE 1. MORTALITY DURING A 4-HOUR EXPOSURE TO ROOM AIR, NITROGEN-ROOM AIR (PLACEBO), OR F-11-ROOM AIR.

Age (days)	Number dead/Number exposed				
	Room Air	7.5% Nitrogen	2% F-11	7.5% F-11	10% F-11
Random Bred	150	0/4	0/4	0/4	--
	240	0/4	0/4	0/4	2/4
Myopathic	150	0/4	0/4	0/4	--
	240	0/4*	0/4**	4/5 [†]	4/4 ^{††}

* Died 3 to 15 days (average 8.1 days) after the exposure.

** Died 4 to 16 days (average 9.5 days) after the exposure.

[†] Four died within 48 min of exposure; the survivor died 2 days later.

^{††} All died within 30 min of exposure

All random-bred hamsters survived 4-hour exposures to room air, nitrogen placebo, and 2% or 7.5% F-11-room air mixtures. Half of the animals died when exposed to 10% F-11 in room air. The survivors of these exposures were alive three weeks later. Asymptomatic, 150-day-old myopathic hamsters also survived exposures of both 2 and 7.5% F-11 as well as air and nitrogen control exposures.

Myopathic hamsters in congestive heart failure (240 days old) did not fare as well during F-11 exposure. Four of the four exposed to 7.5% F-11 died within 30 minutes of the start of exposure and four of five exposed to 2% F-11 died within 48 minutes. The sole survivor died two days later. In contrast, 240-day-old myopathic hamsters survived room air and nitrogen placebo exposures and lived for an average of 8.8 days after the exposure (range 3-16 days).

Having shown that hamsters with heart failure have increased mortality during F-11 exposure, we wondered whether physiologic differences such as electrocardiographic changes between myopathic and random-bred hamsters might also be demonstrable. Cardiac arrhythmias in 120- and 180-day-old pentobarbital anesthetized hamsters were studied during 5 minute placebo or F-11-room air exposures. The incidence of arrhythmias is shown in Table 2.

TABLE 2. CARDIAC ARRHYTHMIAS DURING A 5-MINUTE EXPOSURE TO NITROGEN (PLACEBO)-AIR OR F-11-AIR MIXTURES.

Hamster	Age	Number with arrhythmias/Number exposed				
		7.5% Nitrogen	2.5% F-11	5% F-11	7.5% F-11	10% F-11
Random Bred	120	0/4	0/4	0/4	--	--
	180	0/4	0/4	0/4	0/4	4/4*
Myopathic	120	0/4	4/4**	4/4 [†]	--	--
	180	0/4	1/4 [†]	5/6 [†]	--	--

* Tachyarrhythmia with widened QRS (Fig. 1).

** Shift in QRS axis and variable P-R interval (Fig. 2).

[†] Bradycardia (Fig. 3).

Random-bred hamsters tolerated exposure to as high as 7.5% F-11 without arrhythmias. Ten percent F-11-room air caused a tachyarrhythmia with widening of the QRS complex (Figure 1). Myopathic hamsters developed arrhythmias at much lower concentrations. Five percent F-11 caused severe bradycardia in both 120- and 180-day-old myopathic hamsters. Curiously, 120-day-old myopathic hamsters developed arrhythmias during 2.5% F-11 exposure, and the arrhythmias were different than those caused by 5% F-11 (Figure 2). We observed a shift in the direction of the mean QRS vector in the frontal plane, and a variable P-R interval without any change in the P-P interval or in P wave configuration. This pattern suggest altered atrioventricular conduction (Bellet, 1971). Older myopathic hamsters developed marked slowing of the sinoatrial heart rate without change in the QRS configuration (Figure 3). All of the arrhythmias observed in both controls and myopathic hamsters were rapidly reversed with cessation of exposure, and all animals were alive three weeks later.

A second study to be briefly described was prompted by the controversy following reports by Taylor and Harris (1970) and Harris (1973). We wished specifically to question potential interaction of fluorocarbon 11 and hypoxia in the production of cardiac arrhythmias. The early studies did not resolve this question because of crude exposure methods and difficulty in monitoring arterial oxygen tension in the anesthetized mouse.

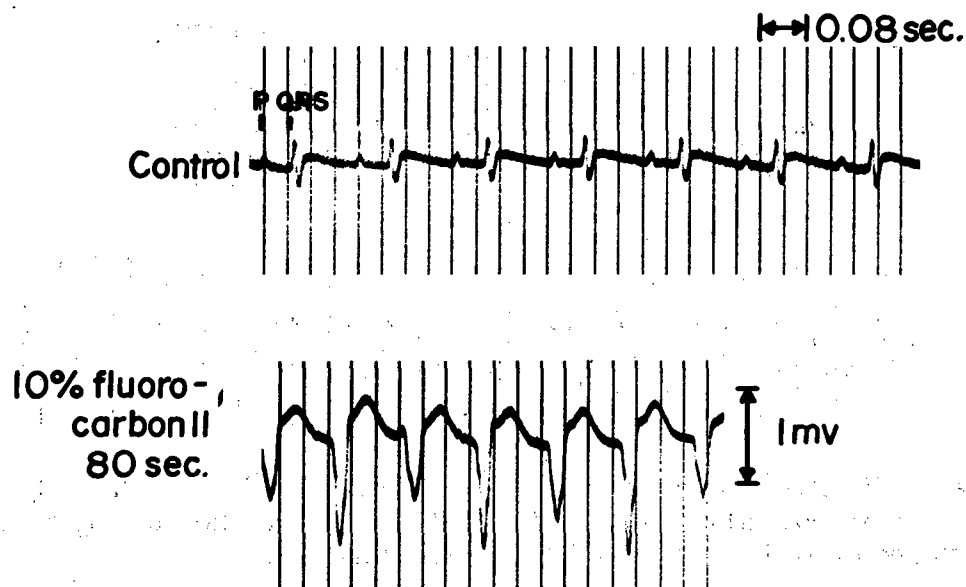


Figure 1. Electrocardiogram (lead II) of a random-bred hamster. (Top) Control, breathing room air. (Bottom) Tachycardia, QRS widening without visible P waves during the 80th second of inhalation of 10% F-11-air mixture.

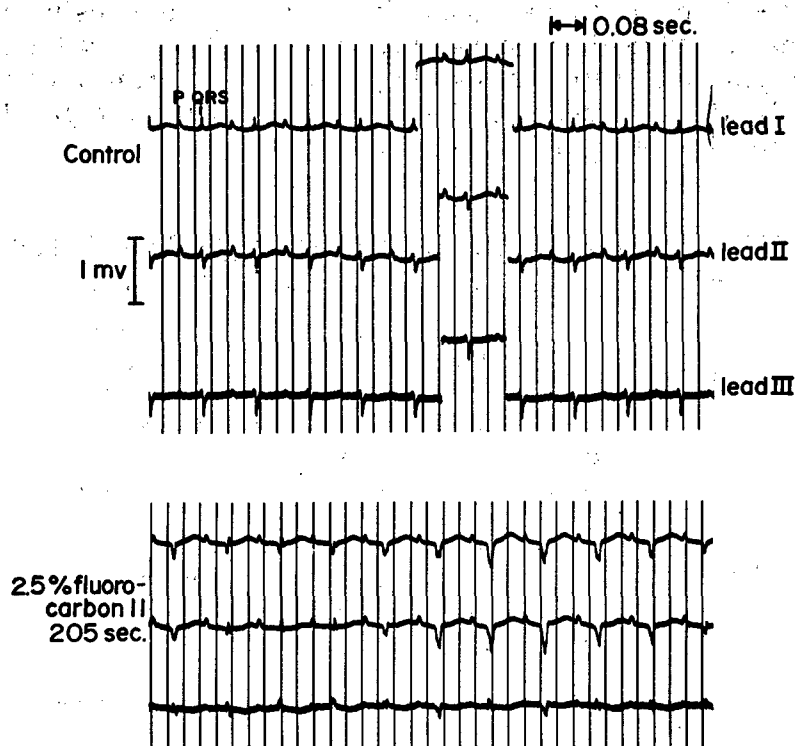


Figure 2. Electrocardiogram (leads I, II, and III) of a 120-day-old, BIO 82.62 hamster. (Top) Control breathing room air. (Bottom) Changing QRS axis with variable P-R interval during the 205th second of inhalation of 2.5% F-11-air mixture.

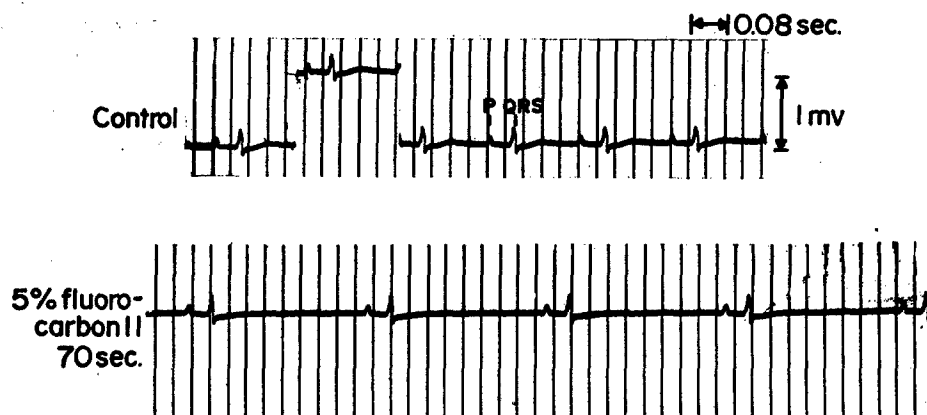


Figure 3. Electrocardiogram (lead II) of a 180-day-old, BIO 82.62 hamster. (Top) Control, breathing room air. (Bottom) Sino-atrial slowing during the 70th second of inhalation of 5% F-11-air mixture.

In the present study, nonanesthetized rabbits had cannulae placed in the middle ear artery. Arterial blood pressure and leads I, II, and III of the electrocardiogram were monitored. The rabbits were exposed to varying mixtures of F-11, nitrogen and room air for four minutes using a nose exposure unit constructed at this laboratory. Table 3 shows blood gases at 2 minutes of exposure and illustrates the protocol. Arterial oxygen tension varied directly with the concentration of inspired oxygen, regardless of the presence or absence of F-11.

TABLE 3. ARTERIAL BLOOD GASES IN 36 RABBITS MEASURED AT 2 MINUTES OF EXPOSURE

	Room Air			11% Oxygen			7% Oxygen		
	PO ₂	PCO ₂	pH	PO ₂	PCO ₂	pH	PO ₂	PCO ₂	pH
Room Air	80	22	7.36	40	27	7.44	32	26	7.37
	100	28	7.40	34	31	7.28	29	29	7.34
	80	25	7.38	42	29	7.44	29	32	7.41
	68	25	7.32	42	30	7.31	28	27	7.46
5% Fluorocarbon 11	85	20	7.40	39	28	7.37	23	23	7.47
	96	26	7.40*	46	28	7.32	29	29	7.29
	63	33	7.33	50	27	7.31	26	26	7.32 ^{†**}
	64	27	7.36	42	30	7.30	31	31	7.32
15% Fluorocarbon 11	80	22	7.38	42	25	7.29	32	22	7.40 ^{†**}
	66	26	7.36	40	19	7.33	33	29	7.34**
	65	27	7.31	46	22	7.35	30	32	7.34**
	70	26	7.32	39	27	7.36**	28	26	7.33**

* Transient premature ventricular contractions.

[†] Recovered from the bradyarrhythmia; 30 minutes after recovery these rabbits tolerated a 4 minute exposure to 7% O₂ without cardiac arrhythmias.

** Bradyarrhythmia during exposure.

Incidence of arrhythmias in this study are those shown in Table 4. At 5% F-11, only one well-oxygenated rabbit developed an arrhythmia, transient premature ventricular contractions, which disappeared by 135 seconds of the exposure. All rabbits breathing 15% F-11-7% oxygen developed bradyarrhythmias, and two rabbits in "adjacent" experimental groups developed similar arrhythmias. Four of these six died; the two who survived were allowed to recover for 30 minutes breathing room air. They were then exposed to 7% oxygen for 4 minutes with no untoward effects.

TABLE 4. INCIDENCE OF CARDIAC ARRHYTHMIAS DURING 4-MINUTE EXPOSURES (N = 36 RABBITS)

	Room Air (range 19.2-21% oxygen*)	11% Oxygen (range 10.5- 11.2%*)	7% Oxygen (range 6.7- 7.5%*)
Room Air	0/4	0/4	0/4
5% Fluoro- carbon 11 (range 4.4- 6.0%*)	1/4 [†]	0/4	1/4*
15% Fluoro- carbon 11 (range 13.9- 16.1%*)	0/4	1/4*	4/4*

* Bradyarrhythmias; four of these six died. After a 30 minute recovery period the two survivors tolerated a 4 minute exposure to 7% oxygen without developing cardiac arrhythmias.

† Transient premature ventricular contractions between 120 and 135 seconds of exposure.

*Concentrations determined by analysis of gas mixtures.

Figure 4 summarizes the effects observed prior to the onset of any arrhythmias. Hypoxia, alone, caused no cardiac arrhythmias but did cause a fall in heart rate without a change in blood pressure. In the absence of bradyarrhythmias, F-11 alone caused a fall in blood pressure and an increase in heart rate. The presence of hypoxia did not alter this response. It is possible that the sinus tachycardia generated by F-11 exposure "over-paced" the atrioventricular or sinoatrial nodes whose conduction had been slowed by hypoxia, leading to missed beats.

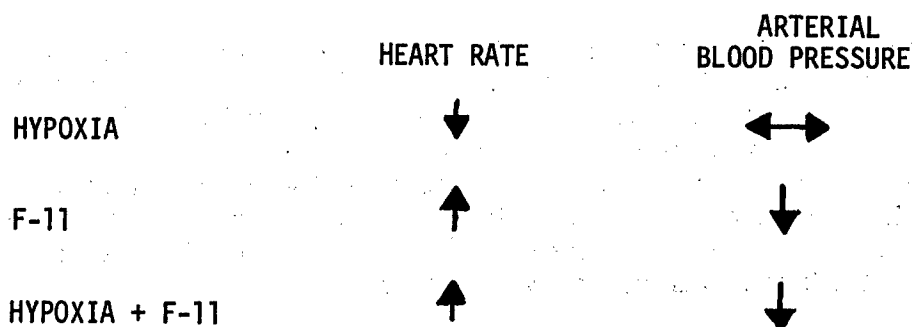


Figure 4. Summary of effects of F-11 and/or hypoxia on heart rate and blood pressure.

SUMMARY AND CONCLUSION

In summary, we have shown that F-11 is more toxic to cardiomyopathic hamsters than to random-bred hamsters, and that the toxicity is qualitatively different as well. Secondly, in another species we have shown that the arrhythmic potential of F-11 is increased by hypoxia and that the arrhythmias observed are not the result of hypoxia alone.

The data from these animal studies are not directly applicable to humans. Genetic cardiomyopathy is not presented as a model of human heart disease or even human cardiomyopathy. The acute hypoxia induced in rabbits is not representative of the acute hypoxic state of the patient with diseases such as asthma or respiratory failure. These data show only that animals with depressed physiologic reserve were more sensitive to F-11 than normal animals.

Interestingly, the diseased animals not only experienced toxic effects at lower doses than controls, but the effects were different. Myopathic hamsters developed atrial arrhythmias and bradycardia while normal animals developed ventricular tachycardia. Similarly, the one well-oxygenated rabbit that developed arrhythmias developed ventricular arrhythmias, while poorly oxygenated rabbits breathing F-11 developed atrial bradyarrhythmias.

Thus, the study of animals with disease or depressed cardio-pulmonary function uncovers new mechanisms of toxicity not seen in the study of normal animals. It may be that the effects observed while studying normal human volunteers may not predict difficulties that those with disease might experience. Obviously, those with disease may be compromised by concentrations of toxins well tolerated by those in good health.

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EFFECT OF RATE OF DOSING WITH ELEMENTAL MERCURY
VAPOR ON BRAIN UPTAKE OF MERCURY

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It is probable that man first had potential exposure to mercury and the potential for developing mercury poisoning when the first man took some cinnabar from the earth. Certainly by the time man separated elemental mercury from mercury ore, there was the possibility of poisoning from excessive exposure to the vapor. Theophrastus of Eresus (371-287 B.C.) is the earliest writer cited by Goldwater (1972) who mentioned cinnabar mines. Goldwater (1972) has provided a review of early writings describing mercury poisoning and cites Agricola and Paracelsus as being the two outstanding contributors to the literature on occupational mercurialism in the 16th century. There is still disagreement on the extent of exposure necessary to cause mercurialism even though it has been known as an occupational disease for over 2000 years and descriptions of the signs and symptoms of the disease have been reported by numerous observers over a period of at least 400 years. Biological variations can account for some of the differences in dose-response that have been reported in the literature on occupational mercurialism; but it appears that there may be errors and misunderstandings that can explain some of the differences.

One of the errors in estimating dose has been in the use of calculated time weighted average exposures based on measurements of elemental mercury vapor in the general work environment. Some of the differences in mercury concentrations in the general work environment and in the micro-environment next to contaminated hands and clothing were reported by Henderson (1972). General work environment measurements of elemental mercury vapor may underestimate the work day exposure by 50 to 90 percent.

Another error in estimating dose of elemental mercury vapor has been the use of the work time only in calculating time weighted average exposure. If hands and clothing are contaminated with elemental mercury, this contamination can be a source of continuing exposure to elemental mercury vapor for the 16 hours off the job. It is conceivable that this off-the-job exposure could be at least twice the exposure on the job and might be infinitely greater. A person working under down draft ventilation and keeping contaminated hands

away from the face during work might have zero inhalation exposure to elemental mercury vapor on the job, then go home and sit with chin in contaminated hands watching television and sleep with contaminated hands near the face resulting in excessive exposure. It is possible to put 10 mg of mercury under one fingernail; vaporized and inhaled that amount is as much as a worker might inhale from exposure to 0.1 mg of elemental mercury per cubic meter of air for 8 hours per day for 10 days.

The possible significance of exposure beyond the work day was reported by Hamilton and Hardy (1949). They wrote, "Some years ago when work was carried on in certain California mines where the ore contains metallic quicksilver, there were miner's wives who became poisoned as a result of washing their husband's overalls. In the mines of Idria children are said to acquire mercurialism if they sleep in the same bed with their father." In the report of the meeting of the International Committee on Mercury (1969) there is the statement, "Contamination of skin or work clothes with mercury compounds, however, could cause heavy exposure to mercury vapor by inhalation." I do not know of a single published report that has estimated the dose of elemental mercury vapor from inhalation both during the work day and for the time away from work. Failure to take the off-work exposure into account may result in gross underestimation of extent of exposure.

Another possible source of error in the understanding of the effects of exposure to elemental mercury vapor may result from considering equivalent time weighted average exposures to be equivalent in effect without consideration of the rate of dosing. Henderson (1972) has discussed the possibility that a rate of dosing by inhalation of elemental mercury vapor in air low enough to permit oxidation of the elemental mercury, as it moves from the lungs and the brain may yield a much lower concentration of mercury in the brain than a slightly higher rate of dosing that does not permit oxidation of all of the elemental mercury as it moves from the lungs to the brain. Table 1 shows the concentrations of mercury in the brains of monkeys exposed to 0.1, 0.5 and 1.0 mg/m³ of elemental mercury vapor 6 hours per day, 5 days per week for 2 to 3 years as reported by Smith (1971).

TABLE 1. MERCURY CONCENTRATIONS IN BRAINS OF MONKEYS EXPOSED TO DIFFERENT CONCENTRATIONS OF ELEMENTAL MERCURY VAPOR IN AIR, $\mu\text{g/g}$ (DRY WEIGHT)

Brain	Control	Vapor Concentrations mg/m ³		
		0.1	0.5	1.0
Medulla	0.1	0.2	24	55
Cerebellum	0.4	0.6	11	64
Occipital	0.2	0.4	15	84
Frontal	0.3	0.6	12	87

The smaller fraction of the total dose that was absorbed from exposure at 0.1 mg/m^3 compared with 0.5 and 1.0 mg/m^3 may be due to the oxidation of a larger fraction of the lower dose before reaching the brain. These data suggest that the rate of dosing may be a factor in the extent of pick-up of mercury by the brain; but since both the rate of dosing and the total dose were different it is possible that the size of the time weighted average dose and not the rate of dosing was the controlling factor.

I had hoped to have the results of an experiment in which animals will be dosed at different concentrations but equivalent time weighted average doses. Unfortunately there has been a delay and all I can report now is the design of the experiment. Rats will be exposed to elemental mercury vapor at concentrations of 0.6 mg/m^3 for 15 minutes, 0.2 mg/m^3 for 45 minutes and 0.1 mg/m^3 for 90 minutes per day, five days per week. Some animals will be exposed for a total of 10 exposures and some for 20 exposures. Urine samples will be collected for determination of elemental, ionic, and total mercury. Blood and brain samples will be analyzed for mercury at the end of the exposure periods. It is hoped that the results may help to answer the question: Should we be more concerned with 15 minutes of high exposure to elemental mercury vapor coming from contaminated hands near the face during a smoking break than with 90 minutes of exposure on the job at a low concentration? The answer to this question may help to explain some of the differences in dose-response reported in the literature on occupational mercurialism.

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CARDIAC SENSITIZATION STUDIES IN DOGS
WITH MYOCARDIAL INFARCTIONS

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INTRODUCTION

Previous experimental studies at this laboratory have shown that fluorocarbons, like many other halocarbons and hydrocarbons, are capable of sensitizing the mammalian heart to an epinephrine challenge, resulting in serious cardiac arrhythmias. These findings suggested that this phenomenon, often referred to as cardiac sensitization is a likely mechanism of death in many of the aerosol-sniffing fatalities which began in the late 1960's.

Certain critical, inspired levels (Reinhardt, 1971 and Reinhardt, 1973) and blood concentrations (Azar, 1973 and Trochimowicz, 1974) of fluorocarbon were associated with the production of cardiac sensitization in healthy beagle dogs. In the present study, dogs with experimentally-induced, myocardial infarctions were tested to determine whether damage to the heart might significantly lower the threshold for cardiac sensitization. This "cardiac model" closely resembles the human condition and can be medically-documented in terms of ECG, drug therapy, biochemical indices, and pathologic examination.

MATERIALS AND METHODS

Materials

The materials studied and their source are listed in Table 1. Methyl chloroform and fluorocarbon 11 are commonly used in industrial operations and are found in many household items - paint products, spot removers, various aerosol sprays, etc. Fluorocarbon 1301 is a gaseous fire extinguishing agent.

Epinephrine (adrenaline) hydrochloride, 1:1000, was obtained from a commercial pharmaceutical company.

TABLE 1. MATERIALS STUDIED

Designation	Chemical Name	Source	Approximate Purity
Methyl Chloroform	1,1,1-Trichloroethane	Dow Chemical Co.	94.5%
Fluorocarbon 11	Trichlorofluoromethane	E. I. du Pont de Nemours and Co.	99.99%
Fluorocarbon 1301	Bromotrifluoromethane	E. I. du Pont de Nemours and Co.	99.98%

Experimental Animals

Forty-one healthy, male, beagle dogs, one to three years old, weighing 7 to 14 kg, from our own colony, were sent to the Cox Heart Institute* where experimental myocardial infarctions were induced by the closed chest technique described below. Thirty dogs survived, were allowed to recover for one to three weeks, and returned to our laboratory. None of these animals were exposed to the test materials prior to five weeks postinfarction.

Infarction Technique

This catheter technique, described in detail elsewhere (Kordenat, 1972) basically consisted of directing a helical wire coil (copper or magnesium alloy), under fluoroscopic control, through an exposed carotid artery downward into the lumen of the circumflex or anterior descending branch of the left coronary artery at selected sites within these vessels. The onset of infarction occurred within several hours following wire placement and depended on the shape and length of the wire. The infarction, resulting from an occlusive coronary thrombosis, was monitored throughout the thrombotic episode in the unanesthetized, intact animal. Various physiological and biochemical parameters were monitored in these dogs before, during, and after myocardial infarction. The exact anatomical location of the wire and the size of the infarction were noted at autopsy during gross examination of the heart.

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Test Procedures

On return to our laboratory, complete electrocardiograms (all leads) were recorded on the test animals every few weeks after their surgery. Since some of the test dogs were exposed to more than one concentration level of the same compound and/or to more than one compound, at least 48 hours was allowed between dog exposures to assure complete fluorocarbon elimination from the body.

Our standard cardiac sensitization test, described in detail by Reinhardt et al. (1971), was used in this study. Basically, it consists of exposing an unanesthetized dog to a certain vapor concentration of test material for ten minutes. The animal receives a control injection of epinephrine (0.008 mg/kg) intravenously five minutes prior to exposure and an epinephrine challenge (same dosage) after breathing the test material for five minutes. Exposure to compound continues for five additional minutes after the challenge dose of epinephrine. Electrocardiographic tracings were taken throughout both control and exposure periods and recorded on Lead I from active electrodes located on either side of the dog's chest, with an indifferent electrode placed over the mid-back region.

Generation, Administration, and Analyses

The desired vapor concentrations of methyl chloroform and fluorocarbon 11 were obtained by injecting a measured amount of compound, using an automatic infusion pump, into a heated air stream of known volume flow. Fluorocarbon 1301, a gas at room temperature, was delivered by metering a volume of the gas from a cylinder and diluting it with a known volume of air. The concentration of test material delivered to the dog was measured every minute during exposure by a standard method of gas chromatography utilizing a flame ionization detector (methyl chloroform and fluorocarbon 11) or a thermal conductivity detector (fluorocarbon 1301). These methods of generation, administration, and analyses have been previously described (Reinhardt, 1971).

Criteria for Marked Response

In evaluating electrocardiographic results, a marked response to the challenge dose of epinephrine considered indicative of cardiac sensitization was the development of an arrhythmia which was not observed following the control dose, and one which was considered to pose a serious threat to life (multiple, consecutive, ventricular beats) or resulted in cardiac arrest (ventricular fibrillation).

All electrocardiograms were read by us and verified by Dr. James W. Buchanan, School of Veterinary Medicine, University of Pennsylvania.

RESULTS

Typical electrocardiographic changes (Lead I) before and after experimental myocardial infarction are shown for one of our "cardiac dogs" (#1226) in Figure 1. The first panel (upper left) shows the normal ECG of the dog with its natural sinus rhythm. Forty minutes after coil implantation (upper right), a deep Q wave and an elevated S-T segment were observed and are indicative of active thrombus formation around the implanted coil. At 24 hours (lower left), the ECG pattern, showing a deep Q wave and absent R wave suggests a ventricular conduction impairment due to myocardial infarction. Even after six weeks (lower right), when scar tissue formation is essentially complete, evidence of infarction is still observed in terms of a deep Q and an inverted T wave. The bottom panel of Figure 1 shows an ECG tracing on the same dog taken during a standard cardiac sensitization test. During exposure to 0.5% (V/V) methyl chloroform, the epinephrine challenge produced a marked response on the ECG in terms of multifocal ventricular beats and a burst of ventricular tachycardia of about three seconds' duration.

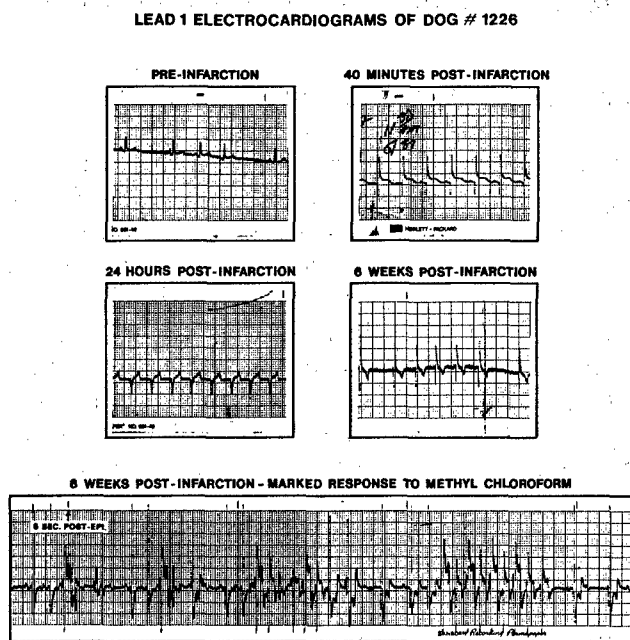


Figure 1. ECGs were taken before and after myocardial infarction at the times indicated: upper left - normal tracing with natural sinus rhythm, upper right - deep Q wave and elevated S-T segment, lower left - ventricular conduction impairment with a deep Q wave and absent R, and lower right - deep Q and inverted T waves. The bottom panel, taken during a cardiac sensitization test, shows multifocal ventricular beats and a three-second burst of ventricular tachycardia.

Aside from ECG changes, various physiological and biochemical parameters can be measured during and after the thrombotic episode in this conscious, unanesthetized intact animal model. One such parameter, for which data are available, is serum glutamic oxalacetic transaminase (SGOT), an enzyme released from the heart in large quantities as a result of myocardial infarction. For example, in 10 of our test animals prior to infarction, the mean SGOT level was 42 units. At 24 hours after coil implantation, the mean SGOT level had risen to 143 units, suggesting that myocardial infarction had indeed occurred.

The results of our standard cardiac sensitization tests, using these cardiac models, are shown in Table 2. The inspired levels of halocarbon selected were similar to those previously tested in normal healthy dogs. The first 15 cardiac dogs received from the Cox Heart Institute were exposed to air alone at four weeks postinfarction. Only one of 15 dogs showed a marked response on the ECG after both the control and challenge doses of epinephrine. Thus, this dog could not be used for subsequent cardiac sensitization testing.

TABLE 2. CARDIAC SENSITIZATION TEST RESULTS
IN DOGS WITH MYOCARDIAL INFARCTIONS

Test Compound	Concentration % (V/V)		No. of Dog Exposures	No. of Marked Responses	Days* Post-Infarction
	Nominal	Analytical*			
Air	-	-	15	1	28 \pm 2
Methyl Chloroform	0.25	0.24 \pm 0.003	12	0	48 \pm 11
	0.37	0.37 \pm 0.006	12	1	53 \pm 11
	0.5	0.50 \pm 0.004	12	4	63 \pm 13
	0.5	0.51 \pm 0.009	12	1	133 \pm 11
Fluorocarbon 11	0.1	0.10 \pm 0.006	12	0	96 \pm 11
	0.5	0.50 \pm 0.01	12	1	98 \pm 12
	1.0	1.00 \pm 0.04	12	6	105 \pm 11
Air	-	-	15	0	22 \pm 4
Fluorocarbon 1301	5.0	5.02 \pm 0.07	10	0	81 \pm 3
	7.5	7.62 \pm 0.09	10	0	84 \pm 3
	10.0	10.09 \pm 0.15	10	2	86 \pm 4

*Mean \pm 1 standard deviation

Of the 14 remaining dogs, groups of 12 were selected at random for exposure to various inspired levels of methyl chloroform and fluorocarbon 11, respectively. Methyl chloroform, tested in dogs at seven to nine weeks postinfarction, elicited no marked responses in 12 dogs at 0.25%, but did produce sensitization in one of 12 and four of 12 dogs at 0.37% and 0.50%, respectively. At 19 weeks postinfarction, the same 12 dogs were again tested at 0.5% methyl chloroform with only one of 12 showing a marked response on the ECG. All of

the marked responses to methyl chloroform consisted of multiple consecutive ventricular beats (ventricular tachycardia) and none resulted in ventricular fibrillation and death.

From the same group of 14 cardiac dogs, at 12 to 13 weeks postinfarction, 12 dogs were selected and exposed to fluorocarbon 11. At 0.1% (ACGIH Threshold Limit Value), fluorocarbon 11 sensitized 0 of 12 dogs tested. However, inspired levels of 0.5% and 1.0% did evoke marked ECG responses in one of 12 and 6 of 12 dogs, respectively. The one marked response at 0.5% was a bigeminal rhythm; the six marked responses at 1.0% consisted of ventricular tachycardia. No fatalities occurred.

With a second group of 15 cardiac dogs, exposure to air alone at three weeks postinfarction produced no marked responses in our standard cardiac sensitization test. Ten of these 15 dogs, at about 12 weeks postinfarction, were selected for exposure to fluorocarbon 1301. Inspired levels of 5.0 and 7.5% evoked no marked ECG responses in any of the 10 test dogs. At 10.0% fluorocarbon 1301, two of 10 dogs did show ventricular tachycardia in response to the epinephrine challenge, but no ventricular fibrillation or cardiac arrest.

Cardiac sensitization tests, at similar inspired levels, had previously been conducted on normal healthy dogs exposed to methyl chloroform (Reinhardt, 1973), fluorocarbon 11 (Reinhardt, 1971) and fluorocarbon 1301 (Du Pont Information Bulletin S-35A, 1971). A comparison of these results with those on cardiac dogs is given in Table 3. For fluorocarbons 11 and 1301, there appears to be no difference between cardiac dogs and normal dogs in terms of "percent" marked response or the threshold inspired level of fluorocarbon needed to sensitize. In addition, in the normal dogs, a number of the marked responses ended in ventricular fibrillation and death - one of two and three of five at 0.5% and 1.0% fluorocarbon 11, respectively, and one of eight at 10.0% fluorocarbon 1301. No deaths occurred in any of the cardiac dogs exposed to either fluorocarbon at the same inspired concentrations.

For methyl chloroform, the threshold inspired level needed to sensitize appears to be similar in normal and cardiac dogs. The "percent" marked responses, at comparable inspired levels for normal and cardiac dogs, is probably the same, also. At 0.25%, there were no marked responses in either the normal or cardiac dogs tested. However, at the only other comparable inspired level - 0.5% - four of 12 (33.3%) cardiac dogs, at seven to nine weeks postinfarction, were sensitized as compared to three of 18 (16.7%) normal dogs. When the same 12 cardiac dogs were retested just prior to sacrifice (19 weeks postinfarction), only one of 12 dogs (8.3%) showed a marked ECG response. Based on the small number of marked responses, the similarity in inspired threshold level needed to sensitize, and the lack of any consistent differences with the other test compounds, these small differences between cardiac and normal dogs at the 0.5% level appear to be insignificant.

TABLE 3. CARDIAC SENSITIZATION RESULTS:
COMPARISON OF CARDIAC* AND NORMAL DOGS

Test Compound	Nominal Concentration % (V/V)	Marked Responses			
		Cardiac Dogs		Normal Dogs	
		No.	%	No.	%
Air	-	1/30	3.3	0/13	0
Methyl Chloroform	0.25	0/12	0	0/12	0
	0.37	1/12	8.3	-	-
	0.5	4/12 ^a	33.3	3/18	16.7
	1.0	1/12 ^b	8.3	12/12	100.0
Fluorocarbon 11	0.1	0/12	0	0/12	0
	0.5	1/12	8.3	2 ^c /24	8.3
	1.0	6/12	50.0	5 ^d /12	41.7
Fluorocarbon 1301	5.0	0/10	0	0/62	0
	7.5	0/10	0	1/18	5.6
	10.0	2/10	20.0	8 ^c /69	11.6

*Dogs with experimentally-induced myocardial infarctions.

^aTested 9 weeks post-infarction.

^bTested 19 weeks post-infarction.

^cOne death.

^dThree deaths.

At the conclusion of these studies (19 weeks postinfarction), 15 of the 30 cardiac dogs were sacrificed and received a gross pathological examination. All dogs showed essentially the same cardiac lesions as seen in Figure 2. This heart (ventral aspect) shows a yellowish-white granuloma (about 2.5 cm diameter) located in the anterior descending branch of the left coronary artery, just below the circumflex branch. The helical coil was found within the granuloma. To the left and below the granuloma, extensive areas of indentation and fibrous connective tissue are seen. A sagittal section through this same heart is shown in Figure 3. Here, the extent of the damage is even more apparent in that a large portion of the left ventricular wall has been totally replaced by fibrous connective tissue. In all of the 15 dogs, the infarcted area involved approximately 25% of the left ventricle, mainly located in the anterior and apical portions.

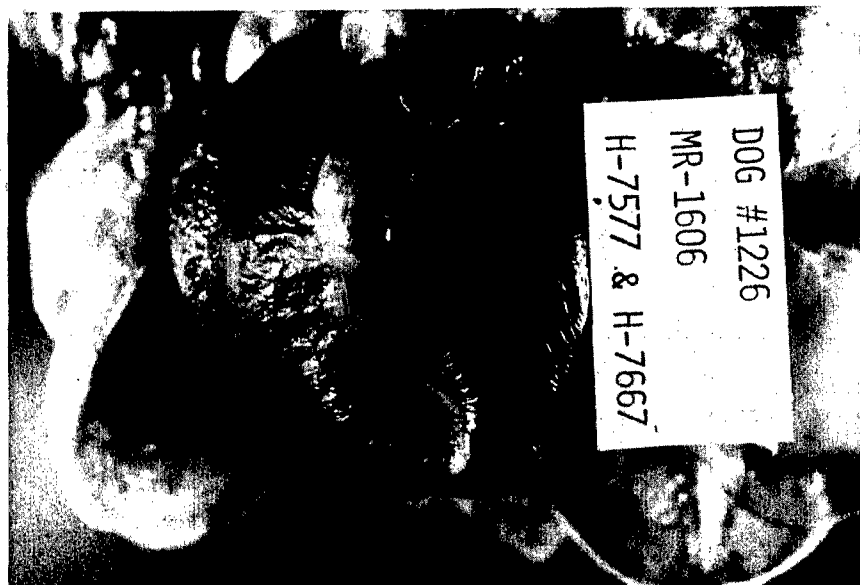


Figure 2. This photograph of a heart (ventral aspect) from Test Dog #1226, at 19 weeks postinfarction, shows a granuloma in response to the helical coil implanted in the anterior descending branch of the left coronary artery (below circumflex branch). To the left and below the granuloma, extensive areas of scar tissue formation and indentation are seen in the left ventricle.

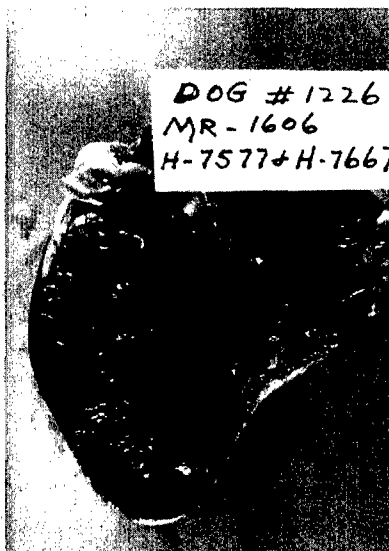


Figure 3. This photograph of a sagittal section of heart from Test Dog #1226 (19 weeks postinfarction) shows a large portion of the left ventricular wall which has been replaced by fibrous connective tissue.

DISCUSSION

In man, myocardial infarction is the most serious form of ischemic cardiovascular disease and, because of its frequent occurrence, it is the single most important type of heart disease. In almost all instances, arteriosclerosis of the coronary arteries underlies the infarction. A thrombus then develops within the vessel, further narrowing its lumen, and finally, complete occlusion occurs.

This disease usually occurs in individuals over 40 years of age, but about 10 to 15 percent of the cases occur in younger individuals. Most infarcts of the heart are found in the left ventricle in its anterior and apical portions, less often in the posterior wall or in the more median portion. Of the main coronary arteries, the highest incidence of coronary occlusion occurs in the anterior descending branch of the left coronary artery.

The model for myocardial infarction used in our present study remarkably resembles the human condition. The sequence of events from thrombosis to infarction, the biochemical and histological events that follow, the coronary vessel occluded, and the site of infarction are all similar. The severity of the myocardial infarction in our dogs was indicated by the 73 percent survival rate and the extent of the heart damage observed at autopsy, as well as biochemical and ECG evidence.

Certain limitations of this cardiac model should also be mentioned. Species differences must certainly be considered in any extrapolation to the human condition. However, because of the histologic and physiologic similarities between dog and man relative to the heart, the dog has been the animal of choice for cardiovascular and anesthesiology research. Another limitation of the model in our particular study is that coronary thrombosis was superimposed on young, normal, healthy dogs whereas humans over 40 years of age with underlying arteriosclerotic disease are the prime candidates for myocardial infarction.

Since myocardial infarction is common in the human population, we attempted to determine whether such a condition might place an individual, exposed to high halocarbon concentrations, at greater risk from an epinephrine-induced cardiac arrhythmia. Such exposure conditions could occur in our own chemical industry, or in the refrigeration and aerosol industries, for example. Using this particular animal model for myocardial infarction, our results do imply that such an individual, having recovered from myocardial infarction and able to return to work, may be at no greater risk from cardiac sensitization than the normal healthy individual.

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CHRONIC TOXICITY OF JP-4 JET FUEL

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Accidental overexposure of workers to JP-4 vapors indicated the need for a Threshold Limit Value (TLV) for this jet fuel based on experimental data. A lack of animal toxicity information necessitated the design of a test to define the chronic toxic effects of low levels of JP-4 vapors on several species of laboratory animals. Toxicity data from this study could then be used either to predict safe exposure levels or provide input for the design of a subsequent test to determine an industrial TLV for JP-4. Application of this TLV would serve to prevent health hazards to those individuals charged with handling JP-4 jet fuel either in storage or in the field.

JP-4 is a complex mixture of aliphatic and aromatic hydrocarbon compounds defined in terms of physical and chemical characteristics, and including various additives, all of which meet the requirements of Military Specification MIL-J-5624E. Those constituents detailed in the military specification are shown in Table 1. Obviously, these constituents represent only a fraction of the total content of JP-4 jet fuel, the remainder consisted completely of unspecified hydrocarbon compounds.

TABLE 1. JP-4 CONSTITUENTS LISTED IN
MILITARY SPECIFICATION MIL-J-5624E

<u>Constituent</u>	<u>Maximum % or Concentration</u>	
Sulfur	0.4	(by wt.)
Mercaptan Sulfur	0.001	(by wt.)
Aromatics	25.0	(by vol.)
Olefins	5.0	(by vol.)
Various Butyl Phenol Antioxidants	24	mg/liter
Aliphatic Diamine Metal Deactivators	5.8	mg/liter

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The American Conference of Government Industrial Hygienists (ACGIH) guidelines indicate that a single TLV for gasoline and/or petroleum distillates is not applicable but that aromatic hydrocarbon content should determine the suitable TLV (Elkins et al., 1963). In essence, the TLV of hydrocarbon-type fuels should be calculated as those vapor concentrations of the fuel which provide 25 ppm benzene (Current OSHA industrial TLV for benzene is 25 ppm).

The toxic effects of chronic benzene exposure to laboratory animals and humans are too numerous to list, but have been well documented and reported by Browning (1965). A summary of the salient hematological and clinical chemical manifestations of chronic benzene poisoning in animals is shown in Table 2. Aksoy et al. (1972) also report an increase in the red blood cell osmotic fragility in humans subjected to long-term benzene exposure.

TABLE 2. SOME HEMATOLOGY AND CLINICAL CHEMISTRY CHANGES ASSOCIATED WITH CHRONIC BENZENE INTOXICATION IN LABORATORY ANIMALS

<u>Increased</u>	<u>Decreased</u>	<u>Other</u>
Lymphocytes	Hemoglobin	Bone Marrow:
Eosinophiles	Red Blood Cells	Erythroid Hyperplasia
Monocytes	White Blood Cells	and Maturation Arrest
Nucleated Red Blood Cells	Platelets	in Erythrocytic and
Reticulocytes	RBC Life Span	Granulocytic Series
Serum Bilirubin	Serum Alkaline Phosphatase	
Serum Lactic Dehydrogenase		

Several acute toxicity tests with JP-4 jet fuel were performed in this laboratory as preliminary to a chronic toxicity investigation. Single oral doses of JP-4 diluted in corn oil administered at 8000 mg/kg produced no deaths in rats. Although sporadic mouse deaths occurred, total mortality could not be achieved at the highest attainable dosage of 1000 mg/kg. A subsequent saturated vapor inhalation test of 6-hours duration to an estimated JP-4 concentration of 38 mg/liter resulted in poor coordination and convulsions in several of the rats, but no mortality.

Gas chromatographic analysis of liquid JP-4 indicated the measured concentration of benzene to be 0.3% by weight. Because of its variable composition, the average molecular weight of JP-4 is unknown. JP-4 concentrations, therefore, are expressed as a fraction of their total hydrocarbon content and measured as mg/liter. Utilizing a Beckman hydrocarbon analyzer, it was determined that the JP-4 vapor concentration which produced 25 ppm benzene contained 5.0 mg/liter total hydrocarbons.

The study, designed to properly assess the inhalation hazard associated with chronic exposure to JP-4 vapors, included four species of laboratory animals exposed to two total hydrocarbon concentrations of the fuel, 5.0 mg/liter (25 ppm benzene), and 2.5 mg/liter (12.5 ppm benzene). Additionally, a positive control group exposed to 25 ppm benzene and an air exposed control group were also maintained. All exposures were intermittent industrial-type exposures of 6 hours per day duration repeated for 5 days per week. The exposure conditions are outlined in Table 3. Test and control groups consisted of 6 beagle dogs (2 female, 4 male); 4 rhesus monkeys (1 male, 3 female), 50 male Sprague-Dawley rats, and 40 female CF-1 mice. Each group of animals was housed in a separate Thomas Dome operated at 40 CFM airflow and 710 mm Hg pressure to avoid leakage of the fuel vapors.

TABLE 3. EXPOSURE CONDITIONS

<u>Dome</u>	<u>Exposure Level</u>
1	UNEXPOSED CONTROLS
2	25 ppm BENZENE (POSITIVE CONTROL)
5	2.5 mg/liter TOTAL HYDROCARBON
6	5 mg/liter TOTAL HYDROCARBON

The JP-4 used in this study was supplied by the Air Force in 55 gallon steel barrels. All fuel received by our laboratory was representative of that found in actual use situations and conformed to the previously listed military specification.

The system used to introduce JP-4 vapors into Thomas Domes is shown in Figure 1. Liquid JP-4 was introduced under pressure from a 55 gallon supply drum. It passed through a glass flowmeter to a heated glass evaporator through which an air supply carried the JP-4 vapors to the exposure dome. Excess JP-4 not vaporized in the evaporator was routed to a receiving tank where it was collected. Thermocouples were placed at the top and bottom of the glass evaporator to sense any increase in temperature indicative of combustion and activate an alarm and solenoid valve system to cut off fuel supply.

Benzene generation for the positive control exposure was achieved simply by use of an infusion pump and glass syringe. Liquid benzene was metered through a "T" fitting to a copper line. Air flowing through the line vaporized the benzene which was then metered through a glass flowmeter to the dome.

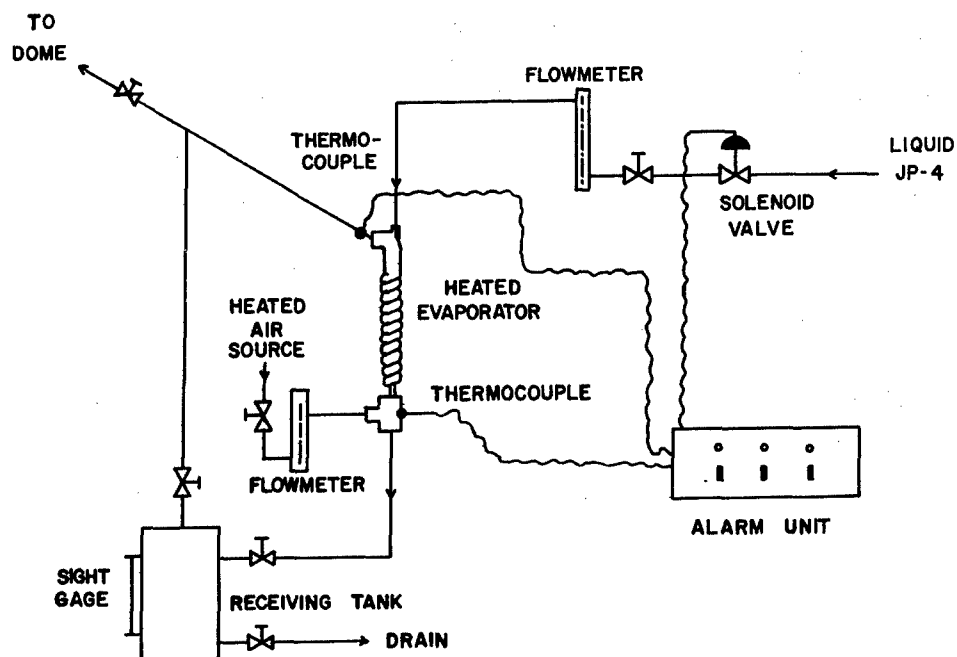


Figure 1. Introduction system for JP-4.

Continuous analyses of JP-4 chamber concentrations were achieved by pumping samples from the dome exhausts into total hydrocarbon analyzers calibrated using known concentrations of propane with the same detection sensitivity as JP-4 vapor. Quantitation of benzene content in JP-4 domes was by gas chromatographic analysis. Benzene vapors from the positive control dome were continuously analyzed using a total hydrocarbon analyzer calibrated with known benzene standards.

The parameters selected to measure the chronic toxicity of JP-4 vapors and effects of benzene at the industrial TLV included biweekly clinical hematology and chemistry tests on dogs and monkeys and biweekly body weights on dogs, monkeys, and rats. The dog and monkey clinical chemistry regimen utilized is shown in Table 4.

TABLE 4. CLINICAL HEMATOLOGY AND CHEMISTRY TESTS
PERFORMED ON DOGS AND MONKEYS
EXPOSED TO JP-4 AND BENZENE VAPORS

<u>Hematology</u>	<u>Chemistry</u>
Hematocrit	Sodium
Hemoglobin	Potassium
Total RBC	Calcium
Total WBC	Albumin/Globulin
Differentials	Total Protein
Mean Corpuscular Volume (MCV)	Glucose
Mean Corpuscular Hemoglobin (MCH)	Alkaline Phosphatase
Mean Corpuscular Hemoglobin Concentration (MCHC)	SGPT
RBC Fragility	

Activity depression in dogs during the initial three weeks of the exposure was one of the two toxic signs noted in the study. The activity of dogs being exposed to either benzene or JP-4 vapors was depressed when compared to their controls. JP-4 and benzene exposed dogs, if not sleeping, were quiescent and prostrate during periods of exposure. The controls, however, remained highly active during a comparable time period. Activity depression in exposed monkeys was evident although not as pronounced as in the dogs. No similar effects were seen in either rats or mice. By the end of the first month of exposure, all dogs and monkeys were exhibiting normal activity patterns. After two weeks of exposure, emesis was noted from one male and one female dog in the high level JP-4 exposed group. The fluid expelled by the male animal contained large quantities of bile while that of the female contained some traces of blood. Emetic activity was not observed again throughout the remainder of the study.

There were no dog or monkey deaths in either the exposed or control groups throughout the exposure. There was one rat and two mouse deaths in both the 25 ppm benzene and the 5.0 mg/liter JP-4 exposed groups. The JP-4 exposed rat and one JP-4 exposed mouse died after 4 months of exposure. The benzene exposed rat was sacrificed during the sixth month of exposure after a mammary tumor ruptured. Two benzene exposed mice and one JP-4 exposed mouse died after six months of exposure. Gross pathological examination of the 5.0 mg/liter JP-4 exposed mouse that died after six months exposure indicated a left lung abscess, pale and blotchy liver, and blood in the abdominal cavity and uterus. With the exception of the benzene exposed rat with the mammary tumor, no gross lesions were noted in any of the other rodents dying from exposure to either 25 ppm benzene or 5.0 mg/liter JP-4 vapors.

The mean body weights of all groups of exposed dogs did not differ significantly from controls at any time during the study. After 12 weeks of exposure, however, a notable but not statistically significant rise in the mean group body weight of the 5.0 mg/liter exposed dogs was observed and continued until the eighteenth week of exposure (Figure 2). There were no significant differences between the growth rates of any of the exposed monkeys when compared to their control group. Exposed rats demonstrated completely normal growth rates for all groups in the study.

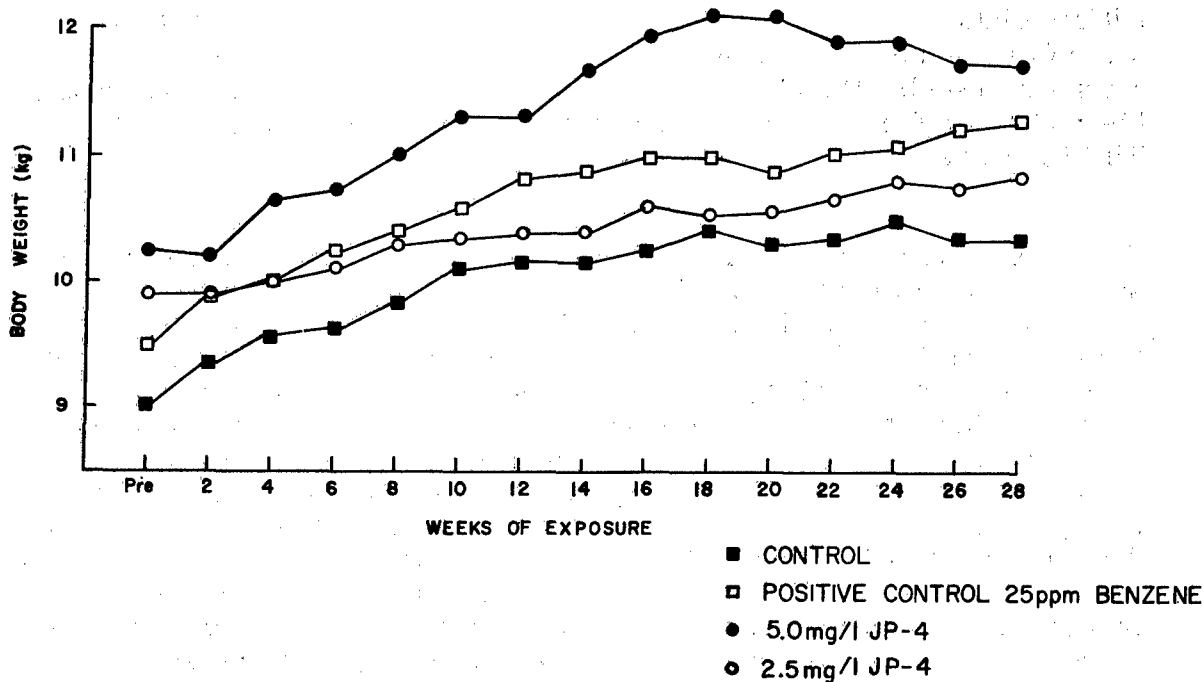


Figure 2. Effect of exposure to JP-4 or benzene on dogs.

Variable sex dependent hematologic effects from chronic benzene exposure have been reported by Browning (1965). There is some supportive evidence to indicate that women and at least two species of female laboratory animals are more susceptible to the effects of benzene than are males.

Biweekly, routine hematological measurements, however, in all exposed dogs and monkeys, both male and female, showed no significant differences from their respective control values. Only scattered values were found to be statistically different from controls and none of these differences was indicative of any trends that could be attributed to either benzene or JP-4 exposure. Blood indices for all dogs and monkeys, calculated from their biweekly hematocrit, hemoglobin and RBC values, did not indicate any difference in test animals when compared to corresponding controls.

Biweekly RBC osmotic fragility testing was performed using a modification of the Davidsohn et al. (1969) method. Initially, only two female dogs and two female monkeys from each dome were sampled. An increase in the RBC osmotic fragility in female dogs exposed to 5.0 mg/liter JP-4 was noted between the twelfth and twenty-second weeks of exposure (Figure 3). The fragility increase occurred between the 0.5 and 0.4% saline solutions used in the fragility assay. There was no evidence of hemolytic effects noted in any of the hematologic parameters tested at the same time the RBC fragility measurements were made. Increased fragility in the 5.0 mg/liter JP-4 female dogs diminished after 22 weeks of exposure and returned to normal by 28 weeks of exposure.

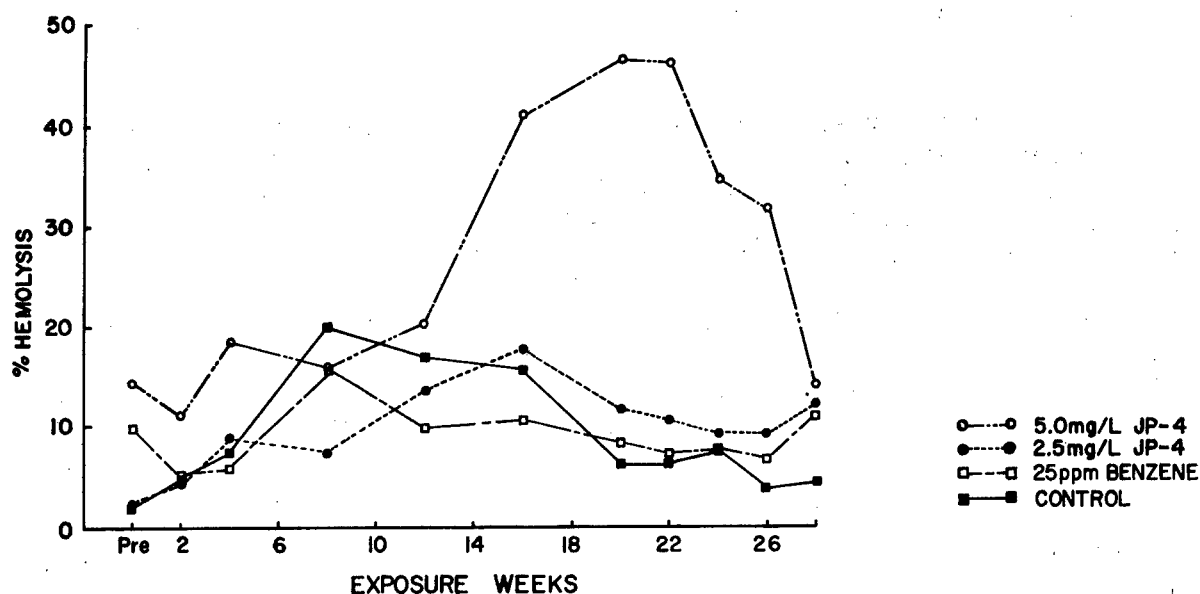


Figure 3. Effect of 5.0 mg/liter JP-4 on ♀ dog RBC fragility at 0.45% saline concentration.

Beginning at 22 weeks of exposure, all beagles were sampled for RBC fragility measurements on a biweekly basis. Table 5 shows the average RBC fragilities for males in each of the three test and control groups, as measured at 0.45% saline concentration, from 22 weeks through 28 weeks exposure. Although the increase in fragility in the 5.0 mg/liter JP-4 female dogs reflected the greatest change from control, there may possibly have been an effect on the male beagles exposed to both concentrations of JP-4 vapor. The males exhibited an increase in fragility over control values; however, there does not appear to be a dose response relationship, i. e. increased fragility in 5.0 mg/liter JP-4 exposed male dogs was not greater than that measured in the 2.5 mg/liter JP-4 exposed male animals. Because data on the male RBC fragility values do not exist before 22 weeks of exposure, it is impossible to assess the fragility effects before this time.

TABLE 5. RBC FRAGILITIES FOR MALE BEAGLES AT
0.45% SALINE

	% Hemolysis			
	Control	25 ppm Benzene	5.0 mg/liter JP-4	2.5 mg/liter JP-4
22 Weeks	4.4	8.8	16.9	18.4
24 Weeks	6.3	12.6	13.7	19.8
26 Weeks	5.6	8.6	6.2	14.7
28 Weeks	4.5	6.9	3.7	9.6

Hematology measurements including hematocrit, hemoglobin, and RBC determinations, done on 5 rats sacrificed from each of the domes at 8 and 16 weeks, failed to show any statistically significant differences from controls. Bone marrow samples from these same animals revealed M/E ratios which were comparable to control values.

Examination of clinical chemistry data, which consisted of 8 separate determinations collected on a regular biweekly schedule for dogs and monkeys, revealed no significant changes in any monkey values which could be attributed to exposure conditions. Dog serum glucose values, however, reflected statistically higher than controls values at 12, 16 and 18 weeks of exposure for the animals exposed to 5.0 mg/liter JP-4 vapors. This rise in serum glucose levels occurred concurrently with the apparent increase in growth rate manifested by the 5.0 mg/liter JP-4 dogs mentioned previously. No other dog clinical chemistry parameters resulted in significant changes which could be related to exposure of either JP-4 or benzene vapors.

This study was extended for sixty days to facilitate the initiation of additional blood studies which would aid in the elucidation of mechanisms accountable for the limited hematologic and clinical chemical changes observed. Three additional assays were included to supplement the routine battery of tests already being performed. These additional tests included methemoglobin determinations on control and 5.0 mg/liter JP-4 exposed dogs, bone marrow studies on iliac crest samples of control and 5.0 mg/liter JP-4 exposed dogs, and red blood cell density distribution determinations on all exposed and control dogs.

The red blood cell density distribution studies and methemoglobin measurements taken on all control and 5.0 mg/liter JP-4 exposed dogs at 26 weeks of exposure failed to demonstrate any difference in test values versus those of the controls. Also, the bone marrow taken from the iliac crest showed no differences between exposed and control values.

Exposure to JP-4 jet fuel vapors has produced increased RBC fragility in female dogs exposed to 5.0 mg/liter concentrations for 6 hours per day, 5 days per week, for up to six months. This same effect was not seen in female beagles exposed to 2.5 mg/liter JP-4 vapors or 25 ppm benzene at the same time periods. Although the increase in fragility does not appear to be a sex specific effect, occurring only in the females, there is a possibility that a similar but reduced effect was also occurring in the males. A conclusive argument for a sex specific effect is precluded by the lack of sufficient data samples for the RBC fragility information. It is our opinion that, although this effect was found in vitro, it apparently had no effect upon the erythrocyte function of the dogs in vivo.

The study was terminated after 33 weeks of exposure. With the exception of 20 rats, 20 mice, and 3 dogs per concentration group, all animals were sacrificed at this time. Gross pathological examination of all species revealed no lesions which could be attributed to exposure.

Organ and organ to body weight ratios for rats have been analyzed statistically and are shown in Table 6. Significant differences from control values were found in organ weights and organ to body weight ratios in the 5.0 mg/liter JP-4 rats. An increase in weight was found in the lung, liver, spleen, and kidney. Micropathological examination of these tissues failed to reveal any dose related effects which could be attributed to this increase in organ weights. I would like to state at this time that we received the pathology report only yesterday and have not had time to examine everything in detail.

TABLE 6. EFFECT OF EXPOSURE TO 5.0 MG/LITER JP-4 ON RAT ORGAN AND ORGAN/BODY WEIGHT RATIOS

<u>Organ</u>	<u>Control</u>		<u>JP-4, 5.0 mg/liter</u>	
	<u>Weight</u>	<u>Ratio</u>	<u>Weight</u>	<u>Ratio</u>
Lung	2.23	0.466	2.38	0.493*
Liver	14.31	2.98	15.75*	3.27**
Spleen	0.90	0.187	1.02*	0.213*
Kidney	3.36	0.700	3.76*	0.782**

*Significant at 0.05 level.

**Significant at 0.01 level.

What appears to be a chemical related effect is the finding of two pulmonary adenomas in 19 mice exposed to 5.0 mg/liter JP-4 vapor. In addition, a mouse was found to have lymphosarcoma in the lungs, spleen, and lymph nodes with metastases to other organs. This mouse was also from the 5.0 mg/liter JP-4 exposed group. No similar lesions were found in the mice from any of the other groups.

As mentioned previously, we have kept rats and mice postexposure from all levels. These animals will be watched carefully and examined at the time of death for pulmonary pathology.

Based on these data, it is suggested that workmen should not be allowed to inhale more than 2.5 mg/liter JP-4 vapors for extended periods of time, i.e., eight hours a day, 5 days per week. It must be emphasized that this standard recommendation is only an estimate based on the available experimental data and is subject to modification following a more detailed examination of the histopathological results.

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TOXIC HAZARDS EVALUATION OF NEW AIR FORCE
FIRE EXTINGUISHING AGENTS

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INTRODUCTION

Low molecular weight halogenated alkanes, particularly certain fluoroalkanes, are of interest to the United States Air Force as fire extinguishing agents, refrigerants, and solvents. Some fluoroalkanes also find use as aerosol propellants and are of interest to industrial and public health agencies because of widespread consumer use of pressurized household products. They also have a potential for abuse, particularly among drug-oriented youth. Another fluoroalkane, halothane, has been in common use in most hospitals since 1954 as an inhalation anesthetic.

Pharmacologically significant exposure of the human organism to these compounds, whether by design or by accident, is usually by inhalation. Many compounds have a relatively high vapor pressure at ordinary conditions, so that they readily mix with air in pharmacologically significant concentrations. Fluoroalkanes readily diffuse through cell membranes because of their lipid solubility. Availability to the alveolar membrane, coupled with lipid solubility, results in a potential for quantitatively significant pulmonary adsorption of fluoroalkanes.

Fluoroalkanes as a rule are not pulmonary irritants. In low concentrations inhalation is not an unpleasant experience acutely, nor does prolonged exposure result in pathological changes in the upper respiratory tract or lungs. In somewhat higher concentrations inspiration may be resisted, but this is likely to be a consequence of the activation of reflexes such as the Kratschmer reflex, as suggested by Aviado.

The USAF is currently most interested in CBrF_3 (Fluorocarbon 1301*), CBrF_2 (F1211), and chlorobromomethane (1011). They are for use in aircraft fire control systems. F1301 is under consideration for deployment in a total flooding system for use in crew and cargo compartments in the C5-A. A combination of F1301 and F1211 has been proposed for use in a new hand-held first aid fire extinguisher, referred to as the "Halonfoam" system. This is under consideration as a replacement for the standard A-20 which uses chlorobromomethane as the fire extinguishant.

METHODS

Dogs were anesthetized with fentanyl-droperidol** and pentobarbital (Hamlin et al., 1968), morphine-chloralose, or ethanol-morphine (Van Stee et al., 1973). Guinea pigs were anesthetized with fentanyl-droperidol and ketamine (0.4 ml/kg and 60 mg/kg, respectively). Monkeys were anesthetized with pentobarbital sodium. Rabbits were killed by cervical dislocation for the removal of hearts for the isolated heart studies, and the rats from which the mitochondria were prepared were killed by a blow to the head.

The general methods for the cross-circulation experiments (Van Stee and Back, 1972), ganglionic transmission studies (Van Stee, 1970; Van Stee and Back, 1972), the cardiac arrhythmia studies (Van Stee and Back, 1971), the Langendorff heart studies (Rusy and Coulson, 1973), and the electron micrographic studies (McNutt et al., 1973), have been reported elsewhere.

The data were analyzed using one and two-way analyses of variance, analysis of covariance, Student's t-test for paired observations, Box's modification of the Bartlett test for homogeneity of variances, and the Mann-Whitney-Wilcoxon test for multiple comparisons when the assumptions underlying the application of parametric methods were known to be invalid (Li, 1964)

RESULTS AND DISCUSSION

Some time ago our attention was directed to F1301 and so it has been subjected to the most detailed scrutiny. Investigation of other fluoroalkanes has followed and we are gradually acquiring enough information to be able to make certain generalizations concerning mechanisms of action and structure-activity relationships.

* Fluorocarbons numbering system: 1st digit, number of carbons; 2nd digit, number of fluorines; 3rd digit, number of chlorines; 4th digit, number of bromines; 5th digit, number of iodines; terminal zeroes are dropped.

** Innovar-Vet, McNeil Laboratories, Fort Washington, Pennsylvania.

Exposure of human volunteers to 15% CBrF_3 was accompanied by irregularities in the electrocardiogram and impressions of impending unconsciousness (Hine et al., 1968; Smith and Harris, 1973). Exposure of monkeys suggested related central nervous system effects. Psychomotor performance of trained monkeys was impaired in the presence of concentrations of CBrF_3 higher than those to which humans have been exposed (Carter et al., 1970). Furthermore, cardiac arrhythmias have appeared in all species tested so far (Van Stee and Back, 1971).

The central nervous system effects of exposure of primates to CBrF_3 differ from those observed in dogs. Whereas in man the effects may be termed depressant on the basis of behavioral observations, dogs become agitated and almost half experience epileptiform convulsions (Van Stee and Back, 1969). It is of interest to note that another fluorinated compound, hexafluorodiethyl ether (Indoklon), has been used in the past for the therapeutic induction of convulsions.

The cardiovascular actions of the fluoroalkanes are considered to represent the most significant hazard incident to their use and so we have conducted detailed studies of the cardiovascular dynamic and myocardial metabolic effects of exposure to F1301, F1211, or chlorobromomethane.

Figure 1 illustrates two consequences of exposure of an anesthetized rhesus monkey to 80% CBrF_3 . A similar picture would be seen during exposure to about 12% of CBrClF_2 . During the exposures, the animals' blood pressure often fell and they developed markedly elevated end diastolic pressures. This indicates that myocardial performance shifted up the Starling curve to a region approaching a state of compensated heart failure which would constitute a decrease of myocardial contractility.

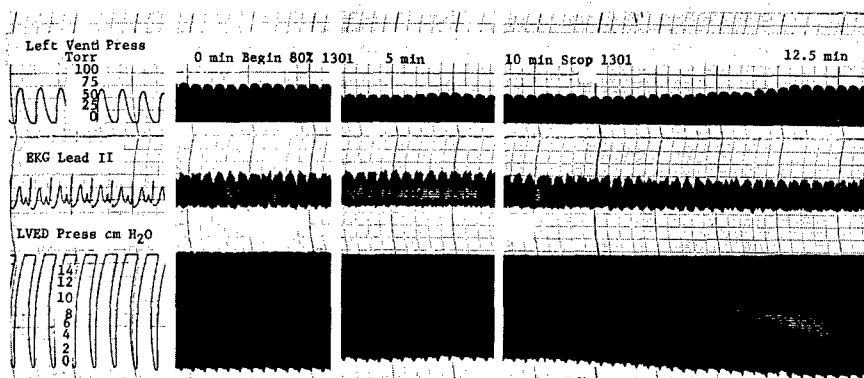


Figure 1. The effect of 80% CBrF_3 exposure on left ventricular blood pressure in an anesthetized, open-chested monkey. The fall in peak systolic blood pressure after 5 min exposure is represented in the top tracing. The bottom tracing represents the same ventricular pressure curve amplified to show the increase in left ventricular end diastolic pressure over the same exposure interval.

The negative inotropic effects of exposure to the halogenated alkanes have been quantified and compared (Van Stee et al., 1973). The decreases in such indexes as dP/dt_{max} and dP/dt_{max} divided by developed pressure were comparable during exposure to approximately 1% 1011, 12-14% 1211, and 75-80% 1301.

The arrhythmias, particularly premature ventricular contractions (PVC), are dependent on a number of factors in addition to the level of fluoroalkane (Van Stee and Back, 1971). Figure 2 illustrates the results of experiments conducted to evaluate the sensitivity of the arrhythmias to mean blood pressure changes. The exposures were constant throughout the periods during which the recordings were made. In the upper tracing the PVC appeared when pressure was elevated by expanding circulating blood volume by infusing 6% dextran. In the bottom tracing PVC appeared and disappeared when mean arterial pressure was raised and lowered by aortic constriction.

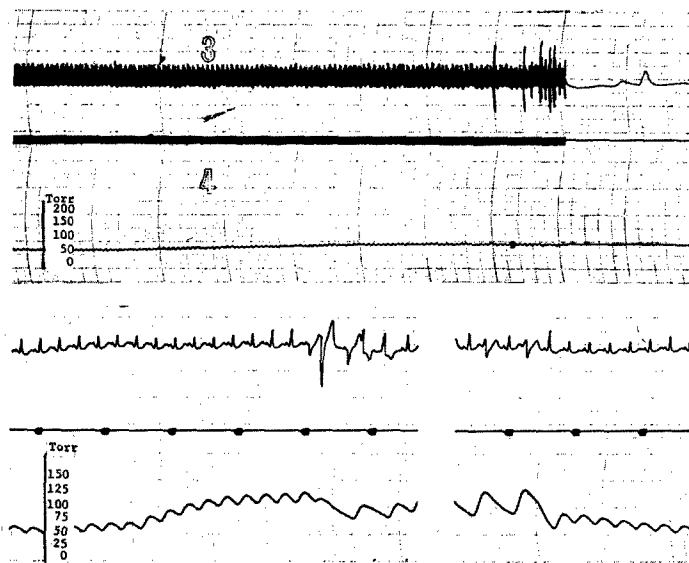


Figure 2. The upper tracing illustrates the triggering of cardiac arrhythmias in a monkey exposed to 70% CBrF₃ by the expansion of plasma volume with 6% dextran. The lower tracing illustrates the triggering and abolition of arrhythmias during a similar exposure by the constriction and release, respectively, of the thoracic aorta.

In another experiment (Figure 3) the blood pressure was lowered and raised by exsanguination and reinfusion. PVC are indicated by the presence of the high peaks in these EKG run at slow speed. Other experiments were conducted that demonstrated that circulating catecholamine levels and acidosis altered the arrhythmia threshold independently of changes in blood pressure.

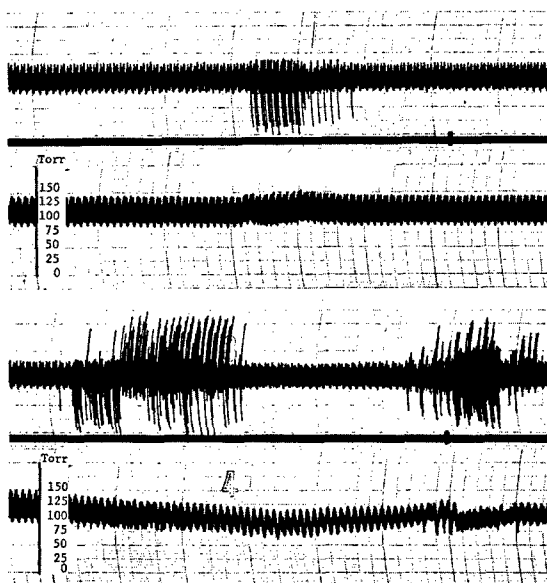


Figure 3. The appearance of cardiac arrhythmias during exposure to CBrF_3 depended on the maintenance of a minimal blood pressure. Arrhythmias (EKG, upper recording of each pair) were triggered by raising the blood pressure (upper pair) with IV epinephrine. Arrhythmias also were abolished and restored by the alteration of blood pressure by exsanguination and reinfusion (lower pair).

Exposure to the fluoroalkanes often caused a reversible, concentration-dependent fall in mean arterial blood pressure (Figures 1 and 5). Cross-circulation experiments have been performed in which the blood from donor dogs was used to perfuse the hind limbs of recipient dogs (Van Stee and Back, 1972). The hind limbs of the recipients were in vascular isolation from the dogs' general circulation but the autonomic innervation remained intact. Through the use of combinations of recipient and donor dog exposures, coupled with the administration of autonomic drugs, the mechanism of hypotensive response to the fluoroalkanes, 1301 and 1211, was determined to be a decrease of vasoconstrictor tone. The compounds were found not to have any direct vascular smooth muscle action.

A series of experiments was conducted to test the hypothesis that the decrease in vasoconstrictor tone was the result, in part, of an impairment of ganglionic transmission (Van Stee and Back, 1972).

The vagosympathetic trunk was severed in the midcervical region and the cut ends stimulated electrically. Nictitating membrane tension was measured during stimulation of the central end and vagal inhibition of the heart was monitored during stimulation of the peripheral end. The fluoroalkanes, but not chlorobromomethane, were found to cause a partial ganglionic blockade.

In another set of experiments intravenous acetylcholine or norepinephrine were the autonomic stimuli (Van Stee, 1970). A reduction in the hypotensive response to injected acetylcholine during exposure to 1301 suggested an anticholinergic effect of this compound.

In summary of the cardiovascular dynamic effects the conclusion was reached that the fall in blood pressure seen during exposure to the fluoroalkanes resulted from a combination of cardiodynamic functional impairment and ganglionic blockade. A reduction of cardiodynamic performance also was seen during exposure to 1011 but since the pressoreceptor reflexes remained functional in the absence of ganglionic blockade, their activation resulted in the maintenance of normal or slightly elevated mean arterial blood pressure during exposure.

The cardiac arrhythmias were sensitive to changes in mean arterial blood pressure which implied that myocardial afterload was a determinant of the arrhythmias threshold as well as the presence of the halogenated alkanes. Tension on the myocardium has been demonstrated to alter both the electrical and mechanical properties of cardiac muscle (Penefsky and Hoffman, 1963). This concept may be extended to include muscle preload and the authors suspect that this variable may affect the arrhythmias threshold as well as afterload. This hypothesis has not been tested in this laboratory but assumes some importance in view of the fact that end diastolic pressure (and presumably end diastolic volume, as well) may rise during exposure to the compounds.

Having determined that the hypotensive effect was primarily the consequence of a decrease in vasoconstrictor tone secondary to ganglionic blockade, a group of experiments was conducted to investigate the mechanism of the negative inotropic effect. Myocardial performance is influenced by factors that might be divided into extrinsic and intrinsic (Figure 4). Each of these must be controlled or eliminated as significant variables in studies of this type.

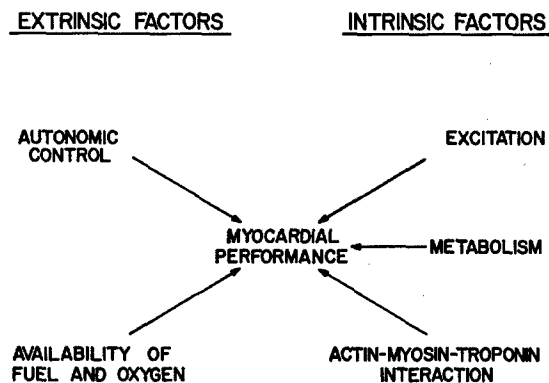


Figure 4. The extrinsic and intrinsic factors that control myocardial performance.

A general procedure was established in which dogs were anesthetized and instrumented for acute exposure to different gas mixtures under anesthesia (Van Stee et al., 1973). Forty variables were either measured directly or computed from measured variables to provide a basis for an evaluation of cardiovascular dynamics and myocardial metabolism during exposure of anesthetized dogs to the halogenated alkanes. The measurements included arterial and coronary venous blood levels of O_2 , glucose, lactate, pyruvate, nonesterified fatty acids, and the acid-base variables. Cardiovascular dynamic variables were monitored and Stewart-Hamilton indicator-dilution studies were performed using indocyanine green.

Some animals were pretreated with amine-depleting doses of reserpine 24 hours prior to experimentation. The results of these experiments indicate that the cardiovascular dynamic impairment that occurred during exposure to 1211 was independent of the integrity of the aminergic neural mechanisms. We have interpreted this to mean that the negative inotropic effect of the compound is independent of myocardial adrenergic postsynaptic activity.

Likewise, the determination was made that the myocardial effects of exposure to the halogenated alkanes was not the consequence of an altered availability to the myocardium of oxygen and oxidizable substrates. Delivery to and extraction by the myocardium of nutrients was not altered significantly.

The significant finding in this series of experiments was that whereas the myocardium was presented with adequate oxygen, the animals exposed to 1211 and 1011 failed to extract a normal amount. A rise in the oxygen content of coronary sinus blood was measured that was correlated with the concentrations of bromochlorodifluoromethane or bromochloromethane to which the animals were exposed. This observation led to an investigation of the role of the intrinsic variables of Figure 4.

More and more evidence has accumulated to reinforce the concept that in the myocardium the primary determinant of coronary blood flow is the tissue oxygen requirement (Dempsey and Cooper, 1972). Coronary flow can, in turn, be a major determinant of myocardial contractile force. As a result of these relationships, when the myocardial oxygen requirement decreases, the coronary flow would be expected to decrease correspondingly, thus maintaining a relatively constant coronary venous blood oxygen content. This relationship was obviously disturbed in the animals exposed to the two compounds in question since as oxygen consumption fell, coronary flow remained undiminished or fell, but coronary venous blood oxygen content rose.

The normal clinical determinants of myocardial oxygen consumption are heart rate, blood pressure and myocardial wall tension (Pitt, 1974). The latter two were demonstrated to be reduced to a certain extent which would account for a reduced oxygen demand by the myocardium but could not account for a normal readjustment of the coronary flow-venous oxygen-myocardial oxygen demand mechanism.

A series of experiments was initiated to study the effects of the halogenated alkanes on the respiration of isolated mitochondria (Sordahl et al., 1971). The equilibration of isolated rat liver mitochondria with the same concentrations of the halogenated alkanes to which the intact animals were exposed resulted in decreases in the rate of respiration without an uncoupling of oxidation from phosphorylation. The order of potency in slowing mitochondrial respiration was the same as that for producing a rise in coronary venous blood oxygen content. CBrF_3 was the weakest, CH_2BrCl was the most effective, CBrClF_2 was intermediate between the two. Given sufficient time, in all cases, all of the ADP added to the isolated mitochondrial suspensions was phosphorylated to ATP.

Since mitochondrial respiration was slowed during exposure to the halogenated alkanes, we conducted a series of experiments to determine the effects of exposure on myocardial ATP content. Anesthetized guinea pigs have been exposed to 50-60% $^{13}\text{O}_2$ in oxygen and their hearts freeze-clamped for phosphate compound analysis. All ATP levels were found to begin to fall after 3.75 minutes exposure. As may be seen in Figure 5, the rise in end diastolic pressure began, in this experiment in a dog, 1.75 minutes after beginning a similar exposure. Mean arterial blood pressure was monitored in the guinea pigs from which the hearts were removed, and the marked hypotensive response to exposure began within a minute of the beginning of the exposure. The evidence clearly fails to support the hypothesis that the cardiovascular dynamic effects are a direct response to myocardial ATP depletion.

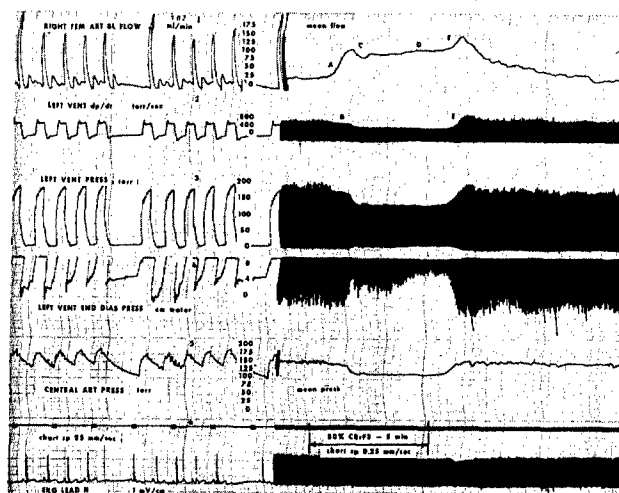


Figure 5. A left lumbar sympathectomy was performed on the dog used in this experiment one week prior to experimentation. Exposure of the dog to 80% CBrF_3 resulted in a large increase in right femoral arterial blood flow. Flow through the contralateral artery (not shown) was unchanged. Concurrently, mean arterial blood pressure, peak systolic blood pressure and $\text{dP/dt}_{\text{max}}$ fell, while left ventricular and diastolic pressure rose. The onset of these phenomena was 1.75 min after beginning the exposure.

The complete significance of the myocardial metabolic derangement has yet to be assessed, but it would appear to be of some biological significance. The salient features are represented schematically in Figure 6.

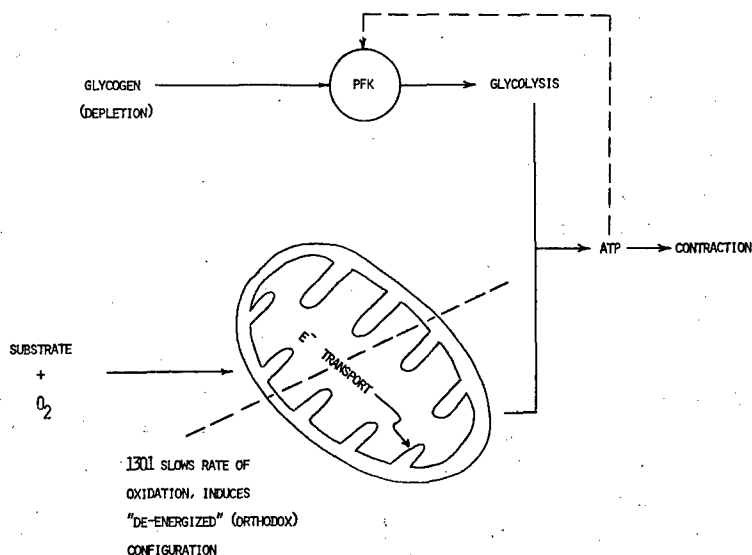


Figure 6. Proposed scheme for the alteration of myocardial metabolism during exposure to halogenated alkanes. The rate of mitochondrial respiration falls which results in a decrease of intracellular ATP levels. ATP is in equilibrium with phosphocreatine (CP) which likewise falls as it is dephosphorylated to rephosphorylate ADP. ATP and CP are normal inhibitors of phosphofructokinase (PFK). When intracellular levels of ATP and CP fall, PFK is activated which results in an acceleration of anaerobic metabolism. A net intracellular decrease in "high-energy" phosphate compounds occurs which contributes to the development of a myocardial performance deficit.

The observation that the rate of mitochondrial respiration was slowed in the presence of the haloalkanes was corroborated by electron micrographic studies of guinea pig hearts exposed to 1301 and perfused in situ (McNutt et al., 1973). The cristal arrangement within the mitochondria of the exposed animals was changed from a condensed to orthodox configuration which was consistent with the phenomenon of slowed respiration. Evidence for hypoxia such as mitochondrial swelling was not detected.

The decrease in the rate of mitochondrial respiration was consistent with the observation by Rhoden and Gabriel (1972) of an activation of myocardial phosphofructokinase (PFK) in rats exposed to 1301. Activation of PFK would be expected to result from a reduced inhibition of this enzyme by ATP and phosphocreatine as they were depleted.

The failure of the detection of an immediate decrease in ATP to account for the decreased myocardial performance led to the study of the effects of the halogenated alkanes on mechanical performance and electrical activity of isolated rabbit hearts perfused with Krebs-Henseleit solution. Preliminary experiments with 1301 and dichlorodifluoromethane, a related compound, indicated that changes in the configuration of the ventricular action potential, conduction velocity, and mechanical activity all were correlated with fluoroalkane level.

The maximal rate of voltage change (dV/dt_{max}) or the slope of phase 0 of the action potential was reduced and the phase 4 plateau abbreviated during exposure to these compounds. The consequences of such alterations of the sarcolemmal electrical activity are represented schematically in Figure 7. Myocardial contractility has been demonstrated to vary with dV/dt_{max} and the duration of phase 2 (Miller and Gilmore, 1972; Morad and Trautwein, 1968; Pruett and Woods, 1967). This is thought to be the consequence of a coupling of calcium translocation to the membrane electrical activity (excitation-contraction coupling). An alteration in sarcolemmal electrical activity during haloalkane exposure could provide the functional basis for the negative inotropic action of these compounds. Furthermore, changes in dV/dt_{max} affect the velocity of propagation of the action potential (Rosen and Hoffman, 1973), a phenomenon measured independently during these studies.

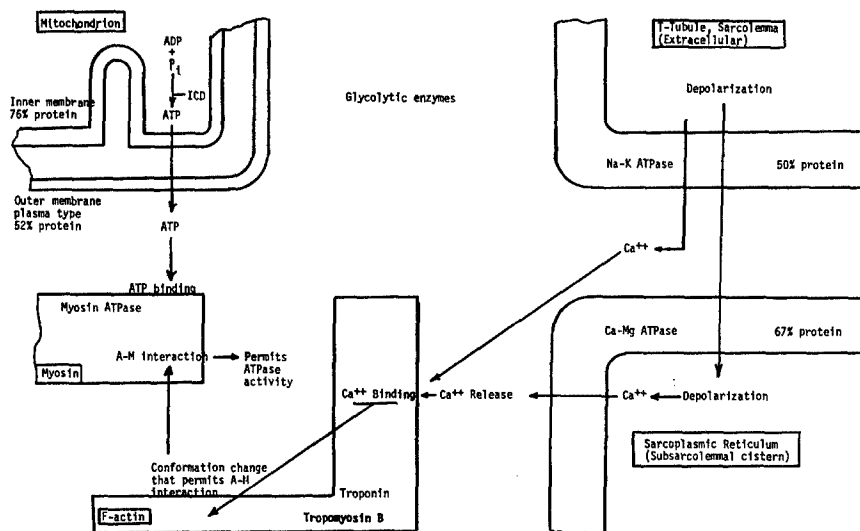


Figure 7. A synopsis of myocardial metabolic and electromechanical events leading to the excitation-contraction sequence. Mitochondrial respiration (upper left) maintains ADP phosphorylation at a rate sufficient to meet the demands of the cell's exergonic processes, principally the actin-myosin interaction (lower left).

The contribution of the changes in electrical activity of the myocardium during exposure to haloalkanes to the genesis of the cardiac arrhythmias has yet to be fully investigated.

The actin-myosin interaction is permitted through an alteration of the configuration of the actin component that accompanies binding of intracellular calcium of troponin. The calcium is liberated from sarcolemmal stores and translocated to the contractile machinery as a function of sarcolemma depolarization (upper right). Relaxation accompanies the reuptake of calcium by the sarcoplasmic reticulum and/or the sarcolemma (lower right).

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OPEN FORUM

DR. MAC EWEN (University of California, Irvine): I'd like to ask a question of Dr. Couri. I recognize that your studies were conducted in an attempt to duplicate the peripheral neuropathy seen in the workers at the plastics formulation plant but I wondered if any studies were conducted to determine the no-effect level, or if any such studies are planned.

DR. COURI (Ohio State University): The reasons we chose the concentrations we worked with are two-fold. First, preliminary experiments showed that the animals will tolerate some of these concentrations for some duration of time as that composite figure showed. The second aspect of this matter was that actual analysis of air samples from the plant indicated that the selected concentrations were similar to those to which the workers were exposed. Now, in answer to your question; we have not followed this up to determine a lower concentration of MBK at which the workers or even animals can be exposed without effects. I think that experiment should be done, but we are not doing it. We are pursuing a more mechanistic approach using the combination of MBK and BK. Butyl ketone seems to enhance the toxicity of MBK in rats and we're interested in the neural toxicity itself. I imagine someone will do studies to determine lower limits. I think we've signalled that the present TLV of 100 ppm which was recently changed from 200 ppm for MBK should be reevaluated. There will be enough attention drawn to this, I think, that someone will follow it up but we will not do it.

DR. MAC EWEN: The reason I asked is that you showed an effect of MBK at 200 ppm, I believe, with no indication of where it stopped. Is anybody here from NIOSH? Are they planning to do any work along this line? Mr. Wands, do you know of any?

MR. WANDS (National Academy of Sciences): I haven't heard of any work. I talked to Dr. Stokinger last week on this briefly and he didn't indicate any active studies underway.

DR. MAC EWEN: Are they considering further revision of the TLV?

MR. WANDS: The last time they met, they were waiting for Dr. Couri's data.

DR. TERHARR (Eastman Kodak Company): I think there are at least two laboratories who are working on identifying what the TLV should be.

DR. MC CONNELL (National Institutes of Environmental Health Sciences): I have one question for Dr. Couri. Did you see any histologic changes in the central nervous system or in any other tissues?

DR. COURI: The only change which I alluded to was one in rats exposed to methylbutylketone both in preliminary studies, where a high concentration was used, and in concentrations which they could tolerate for 14 weeks. By the way, rats showed effects very late, between 12 and 14 weeks, when compared to cats and chickens. They drank a lot more water, roughly twice the values of control animals kept in similar conditions but without solvent exposure. Someone asked the question during my discussion and the question was put, "Are they dehydrated?", and I said, "No, but they drink twice as much water." Of course, that may mean that they are dehydrated. I showed slides of the damage to the sciatic nerve in rats. The same response was seen in chickens as well as cats. The spinal cord and central nervous system showed no histologic changes of this type.

DR. MC CONNELL: The reason I asked was that the lesions you showed looked similar to what has been seen in the sciatic nerve of animals, particularly rats, exposed to methyl mercury. Early investigators were sure that this was a direct effect on the sciatic nerve. In later studies, the investigators found that the damage was actually at the dorsal root ganglion. If you kill the neurons in this location, you will have this Wallerian and axonal degeneration in the sciatic nerve.

DR. COURI: This degeneration was not Wallerian type degeneration.

DR. MC CONNELL: Strictly axonal?

DR. COURI: Yes.

DR. CAVENDER (Becton, Dickinson and Company): I'd like to ask Major Van Stee a question. You stated that you found discrepancies between ATP disappearance and contractility. In correlating the time response, have you considered the fact that creatine phosphate does produce the immediate energy for contraction and the PFK response is to replenish the creatine phosphate?

MAJOR VAN STEE (Aerospace Medical Research Laboratory): I have no doubt, although we didn't take time to investigate that point, that the interval of time between the beginning of the exposure and the point in time at which we see a measurable decrease in ATP probably represents a dephosphorylation of creatine phosphate. We have attempted to set up a method to measure this to see if that, indeed, is what is taking place. We haven't been successful in that method and I doubt very much at this time if we will be able to pursue that in any further detail. Did that answer your question?

MR. WANDS: Two questions. First one for Dr. Couri. Would you comment upon the finding of thiocyanates elevation in the urine? What's the source of the thiocyanate?

DR. COURI: We don't know its source. There were approximately 6 samples that had highly elevated thiocyanate levels out of something like 120 total urine samples. They contained about 10 times what one would consider a normal range. They were well over a milligram per liter which is not even acceptable for heavy smokers. We were operating on the presumption if they were exposed to acrylamide or acrylonitrile or any other product that can yield thiocyanate, we might use this determination as an indirect index of exposure since we were unable to examine the affected workers when they were on the job and exposed to methylbutylketone in addition to other materials. We really had no way to follow it up. It didn't seem like the elevated thiocyanate had significance with so few cases, although those were also ill individuals. They had positive EMG changes and they had some clinical signs of neurological impairment. On the other hand, there were also 60 or 70 other affected workers who didn't have elevated thiocyanates.

MR. WANDS: This could have represented a dietary factor.

DR. COURI: Possibly, yes.

MR. WANDS: I'd like to ask Mr. Kinkead a question. In the production of JP-4 vapors, what temperature did you use for your heating coil and do you think this presented any problem of fractionation? Obviously, the TLV derived is going to be for vapors, not for a mist.

MR. KINKEAD (University of California, Irvine): I'd have to refer to our chemists as far as what the actual temperature was in the evaporator.

MR. VERNOT (University of California, Irvine): It was around 100 F. I'm sure there was fractionation. Unless you vaporize all the JP-4, you're going to get some fractionation. What we were attempting to do was to simulate as best we could the condition to which people working around airplanes would be exposed. That was to produce the concentration of JP-4 vapors that could be volatilized at ambient temperatures.

DR. HODGE (University of California, San Francisco): I'd like to ask Dr. Drew a question. Taylor was the medical student who worked with Dr. Harris once upon a time, is that correct? I think Dr. Harris came to this conference two years ago and told us about some of his experiments.

DR. DREW (National Institute of Environmental Health Sciences): Correct.

DR. HODGE: Do I recall correctly that at that time he considered the effect to be either partially or directly hypoxic?

DR. DREW: Dr. Harris did not consider the effect a direct result of hypoxia but Dr. Silverglade, who also attended that presentation, attacked

the results on the basis that they were caused by hypoxia only. That is what prompted Dr. Taylor to get involved in an actual measurement of the degree of hypoxia.

DR. BUCHWALD (Environmental Protection Services): This is in discussion of the presentation by Mr. Kinhead on JP-4 fuel. In simulating exposures to this fuel, one has to take into consideration possible hypersusceptibility to hemolytic chemicals in the population. I'm not sure that the animal experiment does this, in particular I'm thinking about hypersusceptibility resulting from reduction in activity of glucose 6-phosphate dehydrogenase. The type of thing that was pointed out by Stokinger and Mountain some years ago. I have personal experience of a very tragic case of an individual who died as a result of exposure to the benzene in gasoline. This was an individual who did, in fact, have a genetic hypersusceptibility to hemolytic chemicals. I wonder whether in animal experiments of this kind it is possible to duplicate hypersusceptibility.

MR. KINHEAD: We used 50 rats and 40 mice in each group and they were standard bred animals. They were not selected for any special susceptibility.

DR. BUCHWALD: I mention this hypersusceptibility because there are certain countries, particularly in the Middle East, where the genetic deficiency in glucose 6-phosphate dehydrogenase might be as high as 10% of the population. In a place like that, it might be rather important to study what the effect of the fuel is on hypersusceptible individuals.

MR. VERNOT: The JP-4 concentration which we selected to use in the higher level in the series of exposures was selected on the basis that it provided the TLV concentration of benzene, 25 ppm. Now, it's true that doesn't say anything about hypersusceptibility, but it did at least set the level of the JP-4 vapors to give a concentration of benzene which is considered tolerable in most industrial situations.

DR. JAMES GIBSON (E. I. duPont de Nemours & Co., Inc.): For Dr. Couri. Did you measure other esterases in your experimental animals? I know that you mentioned looking at cholinesterases in the blood of some of the individuals who had been away from work for a while and said that your results were somewhat questionable since they apparently recovered later.

DR. COURI: No, I didn't say they were questionable.

DR. GIBSON: As to their significance?

DR. COURI: No, I said the elevation in the pseudocholinesterase and the diminution in the red blood cell cholinesterase is difficult to interpret. I didn't mean to say they're questionable. Those are solid data as far as I'm

concerned. That's a real effect. The second slide I showed was cholinesterase data from mice, rats and chickens exposed to MEK.

DR. GIBSON: What I'm leading up to here is, did you measure esterases other than cholinesterases, particularly those that are important in myelin synthesis?

DR. COURI: No, we only looked at these two blood esterases. Your point is well taken. We used a radioisotope method and it's very sensitive. You can separate pseudo and true cholinesterase well under that assay. We called this enzyme butyl cholinesterase but I have a suspicion that the Schwann cell has an esterase which probably has very high activity against butyl cholinesterase, and perhaps for cholesterol esters; esters that you would expect to find around nerve damage or actual degeneration.

DR. GIBSON: I'm thinking in particular of those esterase enzymes which are responsible for myelin synthesis; which brings up another question whether the lesion is biochemical or physical action on the axon itself.

DR. COURI: It's really hard to interpret. The best data we have on that point is the EMG analysis and the fact that you can see areas of denodation where the axon seems intact. In fact, often not even swollen, but the absence of myelin is obvious. Myelin does thin sometimes, especially at the swollen portion of the axon. We saw thinning of myelin rather than absence of myelin. And yet, at times you can see in the cytoplasm in the Schwann cell what, by EM staining, looks to be myelin inclusion particles. On this basis, I would like to think of it as a problem related to myelin synthesis or myelin integrity rather than axonal integrity. After all, there is functional activity. These animals can compensate a little and crawl along. And the humans, in fact, have weak legs, but they don't have an absence of function. So there is some motor unit function still prevailing and there is a recovery process. Most of the affected workers have shown recovery on subsequent EMG measurements.

I'd like to ask a question of the floor. This was really our first experience with this kind of thing and we're sort of pushed into the pool. We happened to be in Columbus and this happened in Columbus. The thing that concerns me is when will we get the methods and the techniques to really evaluate subtle and latent toxicities which are a consequence of poor hygienic situations, and poor situations in terms of occupational exposure and environmental exposure. Maybe someone who has a lot more experience can make a comment from the floor about how one anticipates, corrects, or prevents these kinds of things.

DR. HENDERSON (Olin Corporation): I'd like to comment on that point. My first comment would be "physician, heal thyself." I got into a very heated argument recently. We had some respiratory function testing of employees

done in one of our plants by one of our friends at Yale. He chided us because we didn't have baseline respiratory function measurements and he said that he thought every industry had a responsibility to do this. I pointed out to him that I thought every practicing private physician doing physical examinations had the responsibility to recommend this to their patients, rather than shoving it off into industry. I think that this is a part of the problem we are facing today. Where do we get all of this information? We are presently facing this problem with toluene diisocyanate where the proposal is that you shall have baseline pulmonary function testing. I don't know what the facilities are here in Dayton, Ohio: Can you get residual volume measurements? Can you get closing volume measurements? Can you get diffusion rates on 150 employees in a reasonable time and at a reasonable cost in Dayton, Ohio at the present time? I don't know. We're trying to build up the capabilities for this in our medical facility. But for the smaller industries, the smaller businesses, there aren't good industrial medical clinics today that can furnish this type of information. There are physicians practicing curative medicine in industry, but this isn't industrial medicine by any stretch of the imagination. I happen to know a little bit about the Columbus incident, and part of the problem was the fact that they just were not observing their people. I think for them to get to the stage that they were without having somebody take corrective action was very poor industrial hygiene, very poor medical practice. That just doesn't make much sense. This is a total responsibility. It's an across the board responsibility that we look at. This is why we have the Occupational Safety and Health Act. For those of you who aren't familiar with that Act, I would remind you that it says that an employer has a responsibility to tell his employees what the potential hazards are of the materials with which they are working, what the control procedures are, when they're being overexposed and what they are doing about the overexposure. If one wanted to press the point, I think one might say that there was probably a clear violation of the Occupational Safety and Health Act in that installation at the time. The tools for preventive medicine are available at some locations, but there aren't enough of them at the present time.

DR. CULVER (University of California, Irvine): I only want to reinforce what Dr. Henderson says. The work practices as were described in this plant were shocking. Industrial hygienists and occupational physicians have guidelines for the handling of these materials and it appears that this particular plant hadn't heard of these. One other point, Dr. Henderson, is that I think you will find that the practicing medical community will come up with the resources to do the kinds of preemployment examination that is necessary as soon as industry indicates a willingness to purchase those services. I think the problem is to encourage industry to expend some of its financial resources for the human machinery that works inside of a plant as well as for the hardware that works inside of a plant.

DR. HENDERSON: There is a continuing problem of finding competent industrial physicians and competent industrial hygienists at the present time. My company is facing this problem. I don't know what the cutoff point would be, where under the present social climate, you can afford a full time industrial physician in a plant. We've done it in a plant with 600 employees. It wasn't particularly complex. That's pushing the lower limit, I think, of having enough people to keep a good industrial physician busy. There's also a lower limit in plant size for keeping a full time industrial hygienist busy. So you have to, then, have it available either at a corporate headquarters or else if you are not a multi-plant, multi-national corporation, then you've got to be able to go out and buy it in consulting services. It's the smaller plants, I think, that may have some difficulty in buying this type of consulting service at the present time.

DR. COURI: If I may make a comment. I really raised the question conceptually, not to deal with this specific case, but since it has been mentioned, I might as well offer one word in defense for the Columbus medical community. They did, in a very short time, examine 1200 employees at this plant with complete electro-diagnostic neurological testing. They did uncover a large number of common medical problems, a lot of diabetics, a lot of hypertensive people. It was really good community medicine. They did also identify 86 of the workers with abnormal EMG's. The first thing they had to do was establish what a normal EMG should be for that area which apparently was different from the rest of the world and the rest of the literature. So on this basis, they did come through. Somebody did pay for it, obviously. I don't know any physician that would work for nothing. The point is that it was a crisis situation. Why not recognize the problem in the plant initially simply by the stench in the place where the people work. But more so, you say you have to get hit on the head. They began this examination of 1100 or 1200 people only after 6 cases were recognized. So they did respond with the recognition of 6 cases, and it was the workers themselves who suggested that it was probably job related.

DR. HENDERSON: You've given me a soapbox and we've gotten this thing rolling so I'll add another comment. Dr. Couri mentions another effect that I think is important. In a good industrial medical set-up, we are not looking for just industrial diseases. The physical examinations that many of the employees were given may have been their first relatively complete examinations. In this situation, it is quite certain that there will be 20-25% of the persons who will have some abnormality that is not necessarily work related. These may range from previously unknown diabetes to cancer. A benefit then of a good industrial medical program is not only the control of diseases from occupational exposures but is also the early detection and intervention of non-occupational diseases. In many cases early detection and intervention can correct a situation that might be very costly if allowed to continue undetected. This can save on the non-occupational group insurance premiums since these are specifically rated on the basis of what has to be paid out.

The occupational sickness and disability costs in general are on the order of only 10-15% of the total sickness and disability costs for a large corporation. The non-occupational group health insurance costs, the salary continuation costs for a sick person, and death benefit costs are 85-90% of the total costs. A 10% reduction in the non-occupational sickness and disability costs can amount to more than the total occupational sickness and disability costs. A good industrial physician that can keep track of the private physician's treatment of employees can be sure that the dollars expended are actually necessary. A private physician who could discharge a patient after 4 days in the hospital but lets the patient stay a 5th day for the patient's convenience has in essence a 20% excess in hospital costs which may not be medically justified.

The greatest success in control of alcoholism has been in the industrial setting; alcoholism certainly cannot be considered an occupational disease. I expect that we can do the same thing with obesity that has been done with alcoholism; it is well recognized that over-weight is a major medical problem in the United States. In summary, a good industrial medical program should have a far greater impact than merely control of occupational diseases and should complement good private medical practice rather than compete with private practice.

DR. MC CREESH (U. S. Army Environmental Health Agency): Dr. Couri, what is the relative use of methylbutylketone in other industries as opposed to the industry that you are talking about? Are you talking about a large amount?

DR. COURI: It is used in paints, varnishes, lacquer and for many other purposes. It is a very good solvent for cellulose nitrate and cellulose polymers and has wide use. I want to make it clear that this is the first use in plastic coating fabrication where most people have used MEK and MIBK. As a matter of fact, this plant did use MIBK until about a year ago and are now back to using MIBK again. MIBK is a lot less toxic apparently because there have been no new incidences of neuropathy since the removal of MBK.

DR. MC CREESH: Maybe I should rephrase my question. At the end of your presentation, you stated that we must not attribute a cause and effect relationship between the exposure to MBK and the neuropathies that were observed ... that it's more than likely, if I may paraphrase you ... due to a combination of MBK and another compound and/or some other combinations of compounds. Do you have any idea about the concentrations of MBK in this particular plant as opposed to the concentrations encountered in the rest of industry?

DR. COURI: I would guess that MBK here is a lot lower than experienced in other industries, but what I was alluding to is that the process is very extensive; there are conditions of high temperature and humidity and there's a mixture of solvents, solvents with impurities like traces of hexane. I saw one air analysis of 70 ppm hexane in the presence of methylbutylketone, methylethylketone, acetone, diacetyl and aromatic hydrocarbons, which are diluents for other dyes and colors and additives. There were fire retardants, antimicrobial agents and color stabilizers. There's an endless list of at least two or three hundred components. So, we can draw many, many theoretical inferences; the idea of a phosphate compound in the presence of diacetyl could make a nice ring phosphate derivative which would be very neurotoxic. I think those possibilities should be examined. We never got a chance to examine the original material or to use it for animal exposures. That's one of the limitations of this study. I think it's probably a matter of coincidence that MBK happened to be neurotoxic when tested the way we did.

DR. RANADIVE (U. S. Army Environmental Hygiene Agency): I thought it was common knowledge that MBK was the contributory cause. I know of an incident which involved an operation using vinyl paint for painting underwater gates, where they changed the solvent in the paint formulation from MEK to MBK and three out of four painters came down with similar cases of neuropathy over a period of approximately 3 months. They have changed the solvent now. All the rest of the composition of the paint was identical.

DR. COURI: Where is this?

DR. RANADIVE: This was in the southeastern part of the country. The problem was referred to the Army Environmental Hygiene Agency. They have been getting away with sloppy operation until the substitution of MEK with MBK. Addition of MBK to the paint formulation resulted in three out of four painters experiencing identical peripheral neuropathy and all three of them did recover, and now they have stopped using the paint with MBK as a solvent.

DR. COURI: You said identical peripheral neuropathy. You mean that three relative to each other, or do you mean that three when compared to the experience in Columbus?

DR. RANADIVE: Peripheral neuropathy clinically similar to what you described in the Columbus plant.

DR. COURI: That's news to me, but I'd like to make a comment about that. That may simply mean that MBK was the carrier of the toxic agent to this peripheral nervous system. Why should there be this distribution to the peripheral nervous system alone? I think there is a discrepancy in concentration, let's say, in our model animal system relative to the human exposure. One cannot readily explain the distribution of effects. Why only the peripheral nervous system? Maybe you have an explanation for this thing. I still think one might take the position that MBK is an ideal carrier, perhaps for some component of the pigment of the paint which is perhaps common to the plastics industry. You might look at the rest of the composition of the paint involved. I have no particular cause to support or negate MBK as a neurotoxin, but I don't think it's fair to jump to the conclusion that it alone was responsible for the Columbus experience.

MR. WANDS: As Dr. Couri knows, there are many other materials that cause peripheral neuropathies of a somewhat similar nature and particularly demyelination. Triorthocresylphosphate is a classic example, and it is not the active compound. It is a metabolite of the TOCP that causes the injury. It may well be that it is not MBK but a metabolite of MBK that is producing the peripheral neuropathy. I would submit that you should get your biochemists studying this possibility.

DR. COURI: We are studying the metabolites of MBK now. At the present time, we have found in rats, rabbits, guinea pigs, and chickens it's reduced to a hexanol, specifically, 2-hexanol, which is consistent with earlier literature work. Alcohol metabolites were identified, and glucuronides were identified. These studies are still in progress and we haven't done enough to really talk about yet.

DR. CAVENDER: One last question, a very brief one. I wondered if you tested this in neonates to see whether or not the process of myelination itself is affected.

DR. COURI: No, we haven't. That's a good neurological study method but it takes considerable time and effort. There are people who might be interested. I'd say that this problem is far from solved. Not just a question of the causative agent, but the mechanism of action is a fairly exciting aspect of this problem.

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SESSION III

ENVIRONMENTAL CARCINOGENESIS

Chairman

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IDENTIFICATION OF ENVIRONMENTAL CARCINOGENS

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INTRODUCTION

Although approximately 6000 compounds have been tested for long term toxicity or carcinogenicity in laboratory animals, only about 3% of these have been active in animals (National Cancer Institute). Furthermore, in humans only 20 to 30 compounds seem to be environmental carcinogens (Fraumeni and Miller, 1973; J. Weisburger, 1973). However, in the past, it has been the unfortunate fact that the effects of most environmental carcinogens were first detected in exposed persons, rather than in laboratory animals. Therefore, it is of utmost importance both to identify any new compounds which may be carcinogenic in animals and to increase the speed, accuracy, and reliability of the physico-chemical determination of such materials - essentially a problem in analytical chemistry. Examples of possible environmental carcinogens are given in Table 1. Only a few of these are known as carcinogens in humans (Table 2).

TABLE 1. POSSIBLE ENVIRONMENTAL CARCINOGENS

<u>Akylating Agents</u> (mustard gas; industrial intermediates such as dimethyl sulfate, bis(chloromethyl)-ether, ethylene imine, β -propiolactone, epoxides)
<u>Benzene</u>
<u>Drugs</u> (arsenicals; cancer chemotherapeutic agents; hormones; chloramphenicol; diphenylhydantoin; phenacetin)
<u>Dyes and Dyestuff Intermediates</u> (paper and textile dyes; aromatic amines)
<u>Hydrazines</u> (rocket fuels; drugs)
<u>Inorganics</u> (chromates; arsenic; asbestos; nickel carbonyl)
<u>Miscellaneous Intermediates</u> (Dioxane; ethyl carbamate; thiourea)
<u>Natural Products</u> (mycotoxins on foods; plant materials including cycasin, bracken fern, betel nut, wood dust, Senecio alkaloids, safrole)
<u>Nitro-olefins</u> (air pollutants)
<u>Nitrosamines</u> (tobacco smoke; nitrite or nitrate treated foods; endogenous formation; industrial intermediates)
<u>Pesticides, Pesticide Contaminants or Degradation Products</u> (aminotriazole; DDT; 1,2-dibromo-3-chloropropane; ethylene dibromide; ethylene thiourea)
<u>Polycyclic Aromatic Hydrocarbons</u> (soots, tars; heavy mineral oils; air pollutants; combustion products)
<u>Polymer Intermediates</u> (vinyl chloride; certain aromatic amines)
<u>Radiation</u> (sunlight; cosmic; x-rays; fission products; uranium; radium; radon; thorotrast)

TABLE 2. CARCINOGENS IN MAN

<u>Polynuclear Aromatic Hydrocarbons</u>	(soots, pitch, coal tar and products; creosote, shale, mineral, petroleum and cutting oils; cigarette, cigar and pipe smoke)
<u>Aromatic Amines</u>	(2-naphthylamine, benzidine and derivatives, 4-biphenylamine, 4-nitrobiphenyl, auramine and magenta)
<u>Alkylating Agents</u>	(chlornaphthazine, mustard gas, Melphalan, busulfan, bis(chloromethyl)ether, dimethyl sulfate, vinyl chloride)
<u>Nickel Carbonyl</u>	
<u>Chromates, Inorganic Arsenicals, Asbestos</u>	
<u>Radiation</u>	[ionizing, ultraviolet (solar), x-rays, nuclear fission products, uranium, radon, radium, thorotrast]
<u>Mycotoxins, Senecio Alkaloids, Betel Nut, Tobacco chewing</u>	
<u>Diethylstilbestrol (transplacentally)</u>	
<u>Benzene?</u>	

PHYSICO-CHEMICAL DETERMINATION

Within the past 10 years, the development of improved analytical techniques has enabled definite advances in the quantitative determination of chemical carcinogens. A few examples will be discussed briefly.

Hydrocarbons

Of all environmental carcinogens, some of the most widely distributed are the polycyclic aromatic hydrocarbons which appear in soot, tars, smoke, petroleum and combustion products (National Academy of Science, 1972; Hueper, 1963). Their presence often serves as a measure of the degree of pollution of the environment (Shabad et al., 1971). A representative of this class is benzo(a)pyrene, a fairly active carcinogen, which has even been found in forest soils, far from industrial sites (Blumer, 1961). Benzo(a)pyrene is readily determined because of its characteristic fluorescence. Sawicki et al. (1970) have developed a method for benzopyrene in air. The air containing particulate matter is drawn through a filter; the hydrocarbon is dissolved from the particulate, separated by thin layer chromatography on alumina plates, eluted and determined quantitatively by ultraviolet absorption spectrometry or spectrofluorimetry, the latter method being more sensitive. The entire procedure requires only 1.5 hours and the limits of detection are 3 to 200 ng (10^{-9} g).

However, organisms exposed to benzo(a)pyrene metabolize this compound rather rapidly to other substances. Thus a better measure of their exposure may be through detection of metabolites in body fluids or tissues. Recently there have been decided advances in this area through the use of high-pressure liquid chromatography (Selkirk et al., 1974). This technique allows

the rapid and efficient separation of benzopyrene metabolites, including the diols, quinones and hydroxy derivatives, which previously were difficult to separate.

The same method has been employed by Klimisch (1973) to separate the isomers of benzopyrene, a procedure which was extremely tedious otherwise. Additionally, another technique of great value, gas chromatography, is useful for determination of other aromatic hydrocarbons in automobile exhausts or air pollutants (Grimmer and Böhnke, 1972) or of metabolites from other carcinogenic polycyclic hydrocarbons, such as 3-methylcholanthrene (Stoming and Bresnick, 1973). Even the reactive metabolic intermediates such as the epoxides and corresponding diols from 3-methylcholanthrene were separated by this means (Stoming et al., 1973). All these methods are a far cry from the initial laborious fractionations carried out by Kennaway and his colleagues (Kennaway, 1955) to separate and identify the constituents of coal tar.

Aromatic Amines

Certain aromatic amines are further examples of environmental carcinogens; some of them as benzidine or 2-naphthylamine have been implicated as bladder carcinogens in exposed workmen (Scott, 1962). Such compounds have traditionally been employed as dyestuff intermediates, in antioxidants, and to some extent as polymer intermediates or resin hardeners. The possible exposure of workers to benzidine during its production and use as a dye intermediate is obvious. However, if benzidine-derived azo dyes are accidentally ingested, the azo reductase enzyme system can reduce such back to benzidine. Several important paper and textile dyes, from yellows to blacks, are derived from benzidine (Society Dyers & Colourists, 1956-1958).

A problem for regulatory agencies (Occupational Safety and Health Administration, Environmental Protection Agency) is the development of more sensitive assays for benzidine. Udenfriend and colleagues (1972) recently described a new reagent, fluorescamine or Fluoram, which forms a fluorescent derivative with amines that can be detected at the level of 10 to 20 picomoles. However, Fluoram does not give reliable data with benzidine (Rinde and Troll, 1974), but another reagent, 2,4,6-trinitrobenzenesulfonic acid was found more sensitive. The limits of detection are approximately 10^{-9} moles of benzidine. On a volume basis lower amounts can be detected since benzidine is readily extracted from very dilute aqueous solutions by chloroform prior to colorimetric measurement with the sulfonic acid.

A former commercial dyestuff ingredient is 2-naphthylamine. Although it is no longer manufactured for this purpose, it is still an environmental contaminant. Masuda and Hoffman (1969) were able to detect both 1- and 2-naphthylamine in cigarette smoke by means of derivative formation and gas liquid chromatography with an electron capture detector. The smoke of one 85 mm

U.S. nonfilter cigarette contained 27 ng of 1-naphthylamine and 22 ng of 2-naphthylamine. Possible modes of formation are through a thermal reaction of naphthalene and NH_3 in the tobacco smoke or by pyrolysis of amino acids.

Another environmental substance is toluenediamine, an intermediate in polyurethane production. Commercial material is a mixture of about 80% of the 2,4-isomer, a carcinogen in rats (Ito et al., 1969) and 18% of the 2,6-isomer, as yet untested. Analytical methods for toluenediamine, all based on gas liquid chromatography, include derivative formation (Brydia and Willeboordse, 1968) or direct analysis on several types of columns, including the more recently developed carborane-silicone columns (Willeboordse et al., 1968; Yancey and Lynn, 1974).

Alkylating Agents

Several alkylating agents have emerged as environmental carcinogens recently. Bis(chloromethyl)ether is a contaminant of an intermediate used for production of certain ion exchange resins. Besides its potency in experimental animals (Van Duuren et al., 1968), there is epidemiologic evidence for its implication in lung and nasal tumors in exposed production workers (Figueroa et al., 1973). Determination of this compound at a sensitivity of 1 ppb by volume or 4.7 ng/l of air is possible with gas chromatography-mass spectrometry, by estimating the specific ion $\text{C}_2\text{H}_4\text{OCl}^-$ (Shadoff et al., 1973). Vinyl chloride, of great interest, has been linked to angiosarcoma of the liver and other diseases in workers in the polymerization plants (American Chemical Society, 1974). Vinyl chloride detection is possible by gas chromatography at the 1 ppb limit. At the 1 ppm level tentatively suggested by EPA and OSHA as a limit value, several types of portable devices are available based on flame ionization, combustion conductivity or infrared spectrometry for its measurement.

Nitrosamines

Another possible environmental hazard comes from the nitrosamines. These substances, which may form endogenously by reaction of nitrite with weakly basic secondary or tertiary amines or even quaternary ammonium compounds (Wolff and Wasserman, 1972; Sander et al., 1973; Lijinsky, 1974) represent some of the most ubiquitous and universally potent types of chemical carcinogens (Magee, 1974). Besides possible formation in nitrite-treated foods, they occur in tobacco smoke (Johnson and Rhoades, 1972). These compounds, if volatile, can be readily determined by distillation, concentration, extraction, and separation by gas chromatography, followed by confirmation by mass spectrometry (Fazio et al., 1971). The sensitivity is 5 to 10 $\mu\text{g/kg}$ (1 ppb) by this technique. Nonvolatile nitrosamines present a greater problem but liquid chromatography is now showing promise of overcoming this obstacle (Cox, 1973; Heyns and Röper, 1974). However, a recent publication

(Fine et al., 1974) describes a specific detector for the nitroso moiety which obviates the need for extensive clean-up. A methylene chloride solution of any nitrosamine, upon introduction into a flash heater is pyrolyzed, over a heavy metal oxide which acts as a catalyst, to activated nitrogen oxide. In the presence of ozone the nitrogen oxide (NO) forms activated NO_2 . In turn the activated NO_2 decays to NO_2 with emission of light which is detected by a photomultiplier. The response is proportional to the level of the NO grouping in the original nitrosamine, while the sensitivity limits are 0.4 pmole of nitroso compound, equivalent to 30 pg of dimethylnitrosamine.

For all the aforementioned classes of carcinogens, there now exists the possibility of determining their levels or their metabolites in exposed people through the technique of radioimmunoassays. Specific antibodies to molecules as small as nicotine and its major metabolite cotinine have been obtained (Langone et al., 1973). Thereby, it has been possible to detect these two compounds in the picomole range, i.e. < 2 ng of cotinine/ml of serum, in the blood of cigarette smokers. There is no reason why antibodies to other types of environmental substances, for example carcinogens and their metabolites, cannot be developed and employed for estimation of exposure to such materials.

Asbestos

An entirely different situation prevails with the inorganic carcinogen asbestos, a material where the activity depends on the size and shape of the fibers (Stanton, 1974). Despite its environmental importance, there is no rapid chemical method for determination of asbestos. Various schemes are employed to digest away surrounding organic matter, followed by individual detection through x-ray diffraction and electron microscopy (Cook et al., 1974). This is one area where there is a great need for further development of analytical techniques.

BIOASSAY

The biological aspect of identification of possible environmental carcinogens is obviously a much longer procedure than any of the physico-chemical methods described. It involves administration of a compound by the proper route to a sufficient number of the proper animals for a period up to 2 years, followed by thorough histopathologic examination of the animals to detect any abnormalities in relation to controls.

Selection of Compounds for Bioassay

Presently, compounds are selected for bioassay by the National Cancer Institute (NCI) on the basis of usage and production, the number and type of

people likely to be affected, and the structural relationship to known chemical carcinogens. Data from any preliminary studies, information from various government agencies, and input from interested groups are all evaluated before a final selection is made. For each substance chosen, analytical methods are checked or developed. Stability during storage or while mixed in animal feeds is determined before any long-term animal studies are initiated.

Factors Influencing Carcinogenicity Tests

Before selecting a bioassay system, the structure of the substance should be considered, in order to use the material most efficiently. For example, feeding a carcinogenic hydrocarbon in the diet to mice requires large quantities of material but the effect noted is often minimal. Painting such compounds on the skins of mice is a more efficient means of revealing their carcinogenicity. On the other hand, certain aromatic amines have no effect when painted on the skin but yield various types of tumors if fed in the diet or injected subcutaneously (Weisburger and Weisburger, 1967; Arcos et al., 1968, p. 340; Magee, 1970; Health and Welfare Canada, 1973).

The physical and chemical properties must be carefully evaluated. Volatile but nongaseous materials cannot be tested accurately and with safety for animal room technicians if mixed in a diet, but administration of a solution by gavage would be appropriate. Likewise reactive or unstable substances cannot be mixed in diets; administration of a freshly prepared solution is a better method (Shimkin et al., 1966).

Besides the method of administration the dosage and frequency of exposure are important. Saffiotti et al. (1967) found no tumors in hamsters fed 0.1% of 2-naphthylamine in the diet, but at a level of 1% there was a high incidence of bladder tumors. For many aromatic amines a repeated low dose produces tumors, but a single large dose does not (Deichmann and MacDonald, 1968).

The age of the animals often has a measurable influence on a carcinogenicity study. Although the logistics of using newborn animals precludes their routine use for large scale bioassays, they often are more sensitive to many compounds than weanling or adult animals (Vesselinovitch et al., 1972).

The species of animal chosen is most important for certain experimental animals are resistant to various classes of chemical carcinogens although they may respond to others. Guinea pigs apparently lack the enzyme system to form the active carcinogenic intermediate from aromatic amines; if the synthetic preformed derivative was administered they developed tumors (Miller et al., 1964). Although monkeys have not responded to painting the skin with benzo(a)pyrene during periods up to 10 years, they developed hepatomas in 18 months from diethylnitrosamine (Kelly et al., 1966).

Within a given species, there may be a sizable variation in response to carcinogens, either in tumor incidence, the time for tumor development, or both of these factors. Moreover, the spontaneous tumor incidence of the strain of animal should be known. Ideally, the animals should be susceptible to the effects of typical carcinogens but still have a very low spontaneous tumor incidence which simplifies evaluation of the bioassay results. Some strains of animals have very high spontaneous tumor incidence while others, such as the AKR mouse, die from spontaneous neoplastic disease too early to be of value in a bioassay system. Among rats, the Wistar and Sprague-Dawley types have been used a great deal for toxicology studies. They often show a high spontaneous tumor incidence, may be susceptible to respiratory or renal disease and often may reach a very large size, especially Sprague-Dawley males. Simultaneous controls must always be carried for the spontaneous tumor incidence may change over a period of years, as has been noted with Osborne-Mendel and Fischer rats (Weisburger and Weisburger, 1967).

Unless there are very special reasons, bioassay studies should include both sexes for there are differences in their response to various carcinogens. In some cases altering the hormonal situation of the animals can affect tumor response remarkably. An explanation resides in the fact that hormones may control the levels of certain enzymes that activate or detoxify foreign materials such as drugs or carcinogens (Conney and Burns, 1962).

Another important influence is the diet of the animals. It should be nutritionally adequate for restriction in either caloric content or essential nutrients may influence the response to carcinogens. Other extraneous materials, such as mycotoxins, which may themselves be toxic or carcinogenic must be avoided. Pesticide residues can often induce enzymes which increase the metabolic breakdown of foreign materials. Certain plant materials often found in commercially available pelleted diets likewise have some enzyme-inducing capability (Wattenberg, 1971).

Bioassay Routes

For bioassay, compounds may be given orally, either mixed in feed, in the drinking water if soluble and stable, by gavage, or in a capsule. Cutaneous application, useful for testing cosmetic materials, involves repeated painting on the skins of mice or rabbits, taking precautions that the substance is not licked off by the animals. Occasionally, the effect is not evident at the point of application; internal organs or other more susceptible areas may be affected. For gases, aerosols, and similar materials, respiratory exposure either in an inhalation chamber or through a mask covering the face of the test animal, is a pertinent route. Intravenous and intraperitoneal injection require much technical effort but generally afford rapid absorption and distribution of the test substance. These systems simulate the routes by which some drugs are given to patients. Subcutaneous or intramuscular implantation allow accurate delivery of a given dose and are valuable test routes in certain cases, as with

carcinogenic hydrocarbons in mice. With rats there is considerable controversy over the significance of tumors induced at the implantation site.

Protocols and Numbers of Animals

Once a bioassay route has been selected, preliminary toxicity and dose ranging studies, over a 30-90 day period, should be performed with small groups of animals to provide leads for the long term study. By following the food consumption, weight gain and general condition of the animals, a reasonable judgment on the final doses is feasible. In the definitive study, it is most desirable to use more than one dose level, not only to yield dose-response information, but also to serve as insurance in case delayed toxicity develops in animals on the higher levels after several weeks or months of the study. Present NCI protocols call for starting at least 50 animals of each sex and species at each of 2 dose levels, a total of 400 animals if both rats and mice are involved. Sufficient control animals, kept under similar conditions as the experimental ones, are necessary.

In order to check that the animals used are not resistant to carcinogens, it is desirable that a small group be given a known carcinogen. With the advent of OSHA regulations covering several of the usual laboratory-type carcinogens, additional safety precautions are required for such efforts. However, it is still prudent to check the response of the species and strains employed every 2 or 3 years, the time generally needed for a thorough carcinogenicity test.

During the course of the experiments attention to proper animal husbandry practices will generally afford better survival of animals throughout the experiment. At the end of the experimental and/or holding period, preferably at least 2 years for rats and 18-20 months for mice, both the experimental and control animals should be killed for histopathologic study. Careful necropsies by well-trained technicians, preferably under the direct supervision of an experimental pathologist, are absolutely essential. Under current NCI protocols, most organs plus samples of spinal column, vertebrae, and the like are fixed for histopathologic examination. Microscopic examination of tissue sections must be done by a pathologist with experience in classification of animals lesions so that non-neoplastic, precancerous and neoplastic conditions can be differentiated. An automated or computerized system allowing rapid retrieval of information is a necessity with a large scale bioassay system to record all the data efficiently. Finally a statistical analysis of the data is necessary to define the significance of any difference in tumor incidence between experimental and control animals (Snedecor and Cochran, 1967).

The bioassay procedure, which may cost from \$80,000 to \$100,000 per compound, often requires at least 3 years before all the evaluations are completed. For these reasons, there is much interest in developing prescreening techniques which are simple, more rapid and much cheaper. Although they do not substitute for the long-term studies, they may help select those compounds

of a series which are more likely to be suspicious. Some of the methods are: transformation of various types of cells in culture, evaluation of mutagenic effects in bacteria, yeasts or mammalian cells, effect on DNA repair, and also combinations of in vivo and in vitro studies (Di Paolo et al., 1973). Previously, many other rapid screening tests such as tetrazolium reduction by hairless mouse epidermis, photodynamic toxicity in paramecia, cell proliferation, sebaceous gland suppression, effects in brine shrimp, newts and other lower organisms have been proposed (Weisburger and Weisburger, 1967; Arcos et al., 1968). Thorough evaluation of all these methods is required to decide whether there is a true correlation between the effects and carcinogenic activity. One must remember that cells in culture, bacteria and the like may not have the detoxification pathways and immunological defenses of the mammalian organism. Prescreening studies can give us leads, and a battery of them may prove useful in predicting long-term results. However, the final decision on the carcinogenicity of any compound still rests on the effects in animals.

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MECHANISMS OF CARCINOGENESIS BY NITROSO COMPOUNDS

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Since the first report of the carcinogenic action of dimethylnitrosamine by Magee and Barnes in 1956, a large number of N-nitroso compounds (Figure 1) has been shown to induce tumours in a wide range of different species including birds, fish and amphibia as well as mammals (Table 1).

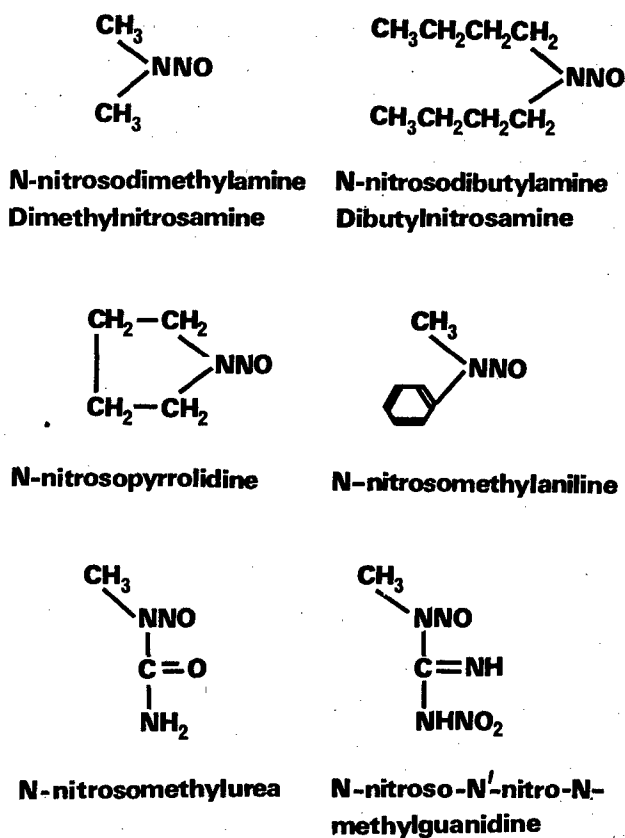


Figure 1.

TABLE 1. SPECIES SUSCEPTIBLE TO CARCINOGENESIS
BY NITROSAMINES

<u>Mammals</u>	<u>Birds</u>
Monkey	Fowl
Rat	Grass parakeet
Mouse	
Guinea Pig	<u>Fish</u>
Syrian Hamster	Rainbow Trout
Chinese Hamster	Aquarium fish - <u>Brachydanio rerio</u>
European Hamster	
Rabbit	<u>Amphibia</u>
Dog	Newt - <u>Triturus helveticus</u>
Pig	
Hedgehog	

Aspects of nitrosamine carcinogenesis have been discussed in several reviews (Druckrey et al., 1967; Magee and Barnes, 1967; Magee et al., 1975). The sites of tumour induction by different nitroso compounds may vary markedly with their chemical structures. For example, dibutyl nitrosamine (Figure 1) and 4-hydroxybutylbutyl nitrosamine are the only dialkyl nitrosamines reported to induce bladder cancer in experimental animals, and N-nitrosomethylaniline (Figure 1) appears to be specific for the oesophagus. N-methylnitrosourea induces tumours at many sites (Leaver et al., 1969) including the nervous system (Druckrey et al., 1967) and N-ethylnitrosourea has a remarkable degree of specificity for this system, particularly after administration by the transplacental route (Ivankovic and Druckrey, 1968). This organ specificity of the nitroso carcinogens has been described by Druckrey as organotropism (Druckrey et al., 1967). The nitrosamines have not been found to induce tumours at the site of application, e.g., the skin or subcutaneous tissue, while some nitrosamides are locally active.

Much of the investigation on mechanisms of action of the nitroso compounds has been done with two of the simplest compounds, dimethylnitrosamine (N-nitrosodimethylamine) and N-methylnitrosourea (Figure 1). Dimethylnitrosamine, like other dialkyl nitrosamines, is powerfully hepatotoxic, inducing severe haemorrhagic centrilobular necrosis in the rat (LD₅₀ about 30-40 mg/kg body wt.) and other species (Barnes and Magee, 1954). The action on the liver is remarkably specific with relatively less severe acute damage to other organs. When fed to rats at dose levels of 50 ppm in the diet a high incidence of tumours in the liver is induced (Magee and Barnes, 1956) and even dietary levels as low as 2 ppm have been reported to be hepatocarcinogenic (Terracini et al., 1967). Feeding relatively high levels of dimethylnitrosamine to adult rats for periods up to a month or large single doses (near to the LD₅₀) does

not cause liver tumours but tumours of the kidney. With single doses renal tumours are found in about 20% of the animals surviving for 8-9 months or more (Magee and Barnes, 1962). This induction of renal tumours is of interest because the nitrosamine is eliminated from the bodies of the rats within the first 24 h after administration, largely by metabolism (Heath, 1962). The balance of the available evidence indicates that dimethylnitrosamine, and probably other dialkylnitrosamines, require enzyme-mediated conversion into carcinogenically active metabolites. It has been found that brief exposure to protein-deficient diets reduces the toxicity and the rate of metabolism of dimethylnitrosamine (Swann and McLean, 1971) and that renal tumours can be induced in all the animals surviving the larger single dose (60 mg/kg body wt.) of the compound (McLean and Magee, 1970). This model has been used by Hard and Butler (1970a & b, 1971a, b, c & d) in a very detailed light and electron-microscopical study of the origin and development of these renal tumours. The tumours are of two distinct types, one obviously of epithelial origin and the other derived from mesenchymal cells. Opinion is divided on the nature of the mesenchymal tumours, Hard and Butler (1971b) concluding that they are of vascular origin. These authors concluded that cells with the morphological characteristics of the mesenchymal tumours were present in the kidneys of the treated rats within the first week after treatment and observed cellular reactions to these apparently neoplastic cells which they thought represented an immunological response. More recently the growth of these early tumour cells in cultures made from the kidneys within the first week after treatment has been reported (Hard et al., 1971). In addition to having the morphological characteristics of neoplasia the cells isolated from treated rats as early as 20 h following treatment had a prolonged life-span in vitro and an increased mitotic index, produced colonies in methylcellulose gels, showed a high relative plating and growth efficiency and were agglutinated in the presence of Concanavalin A (Borland and Hard, 1974). These observations, if confirmed, are clearly of great interest for the understanding of the mechanisms of malignant transformation in vivo.

Although no liver tumours appear to have been induced in adult rats following large single doses of dimethylnitrosamine, such tumours have been induced in animals treated in the neonatal period (Terracini and Magee, 1964). This observation suggested a possible role of concurrent cell division in the induction of liver tumours and led Valda Craddock to examine the effects of single doses of dimethylnitrosamine after partial hepatectomy in adult rats. Administration of dimethylnitrosamine at various times after operation resulted in haemorrhagic hyperplastic nodules and trabecular carcinomas appearing 1-2 years later, the maximum incidence of liver cell carcinomas occurring when the carcinogen was given 24 h after hepatectomy. The author concluded that her results were consistent with the view that replicating cells are specially sensitive to the action of the carcinogen (Craddock, 1971). Liver tumours were also induced when N-methyl-N'-nitro-N-nitrosoguanidine was administered after partial hepatectomy but not after treatment with methyl

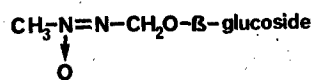
methanesulphonate (Craddock, 1973). Results of a similar kind were obtained by Thomas and Bollmann (1974) who obtained a 100% yield of thyroid tumours in rats after combined administration of N-nitrosomethylurea and methylthiouracil while none appeared in animals receiving the same treatment with the nitrosamide alone.

Metabolism of dimethylnitrosamine occurs mainly in the liver (Magee, 1956) and the production of more of the active metabolite in this organ may explain the greater damaging action in the liver than in the other organs. In vitro metabolism has been demonstrated by liver slices and homogenates and the activity is mainly in the microsomal fraction with requirements for O_2 and reduced pyridine nucleotides. The rate of metabolism by microsomal preparations was slow (Magee and Vandekar, 1958). More recently McLean and Day (1974) have reported that microsomal demethylation of dimethylnitrosamine occurred at only about 1/50 the rate with aminopyrine as substrate and described new methods for its measurement. Metabolism of dimethyl and diethylnitrosamine by slices of rat and hamster tissues in vitro has been studied by Montesano and Magee (1974). These workers found the highest rate in the liver preparations from both species with the dimethyl compound and very low rates in small intestine and intermediate levels in the kidney of the rat. Diethylnitrosamine metabolism, as measured by $^{14}CO_2$ production, was remarkably high with hamster lung preparations. These results give support for the hypothesis that the organ specificity of the nitrosamines is determined to some extent by the capacity of an organ to convert them into carcinogenically active metabolites since dimethylnitrosamine is carcinogenic in rat liver and diethylnitrosamine induces tumours in hamster lung.

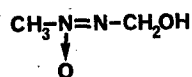
The nature of the active metabolites of the nitrosamines is not known with certainty although there is good evidence for the formation of alkylating intermediates from several of the compounds. Denitrosation of N-methyl-N'-nitro-N-nitrosoguanidine in vivo has also been reported (Kawachi et al., 1970). Alkylation of nucleic acids and proteins in vivo has been demonstrated with dimethylnitrosamine (Magee and Hultin, 1962; Magee and Farber, 1962; Craddock and Magee, 1963; Swann and Magee, 1968; Turberville and Craddock, 1971), diethylnitrosamine (Swann and Magee, 1971), N-nitrosomorpholine (Stewart et al., 1974) and other dialkylnitrosamines. The nitrosamides N-methylnitrosourea (Swann and Magee, 1968) and N-ethylnitrosourea (Swann and Magee, 1971) have also been shown to alkylate in vivo. The evidence for alkylation of nucleic acids in vivo in the earlier work was based on demonstration of radioactive 7-methylguanine in 1M acid hydrolysates of nucleic acids from tissues of animals treated with labelled nitrosamines. It was not until the work of Loveless (1969) that it was appreciated that hydrolysis of nucleic acids under these relatively strong acid conditions caused destruction of guanine alkylated on the O^6 -position. Recent work, using acid of lower concentration or enzymes for hydrolysis, has led to a new approach to research in this field. The biological effects of alkylation of N^7 in guanine are thought

to be less important than those of alkylation on O⁶ because substitution in the latter position did not affect the coding properties of synthetic polynucleotide templates for RNA polymerase (Ludlum, 1970) while O⁶-alkylation has been shown to result in miscoding (Gerchman and Ludlum, 1973). Recent work by Goth and Rajewsky (1974a & b) has underlined the possible significance of O⁶-alkylation of DNA in vivo. These workers studied the persistence of O⁶-ethylguanine in neonatal rat brain DNA after treatment with the nervous system specific carcinogen N-ethylnitrosourea. Similar initial degrees of DNA ethylation were found, in terms of reaction at N⁷ and O⁶ of guanine in brain, a target organ, and liver, a nontarget organ. However, the elimination rate from DNA of O⁶-ethylguanine was strikingly slower in brain than in liver and also much slower than the rate of elimination N⁷-ethylguanine from the brain. The authors suggest that the rate of elimination of O⁶-ethylguanine from DNA, in conjunction with the requirement for DNA replication of the target cell, may be an important factor in carcinogenesis by N-ethylnitrosourea. Comparable results have been obtained by J. Nicoll and A. E. Pegg (personal communication) who compared rates of removal of O⁶-methyl- and N⁷-methylguanine from the liver and kidney in rats treated with dimethylnitrosamine.

Cycasin (Figure 2), the glycoside of methylazoxymethanol, is a naturally occurring carcinogen found in Cycad plants. The patterns of acute toxicity and carcinogenesis induced by the glycoside and its aglycone are remarkably similar to those resulting from administration of methyl nitroso compounds and there is evidence of methylation of nucleic acids in cycasin treated rats (Shank and Magee, 1967; Nagata and Matsumoto, 1969). There is, however, a fundamental difference in the mechanisms of activation of the two compounds since cycasin is not activated by tissue enzymes but by β -glucosidases present in the microbial flora of the intestinal tract (Laqueur and Spatz, 1968). 1,2-Dimethylhydrazine is a potent and specific inducer of tumours of the colon and rectum in rats (Druckrey et al., 1967b). In contrast 1,1-dimethylhydrazine and monomethylhydrazine appear not to induce colonic or rectal tumours, although they have been reported to induce tumours elsewhere which are often of vascular origin (Toth and Wilson, 1971; Toth, 1972). All three methylhydrazines are metabolised by rat liver microsomal enzymes in vitro and methane and formaldehyde have been identified as products (Dost et al., 1966; Prough et al., 1969, 1970). 1,2-Dimethylhydrazine was shown to methylate nucleic acids of liver and colon in rats and mice to an extent comparable with that found with N-methylnitrosourea while monomethylhydrazine had only extremely low activity (Hawks and Magee, 1974). No evidence was found of methylation of nucleic acids prepared with 1,1-dimethylhydrazine in vivo (Kruger et al., 1970). It has been suggested that 1,2-dimethylhydrazine acts in a similar way to the nitrosamines and cycasin and differently from the monomethyl derivatives (Hawks et al., 1974).



Cycasin



Methylazoxymethanol



1,2Dimethylhydrazine

Figure 2.

There is widespread interest in the possibility that nitrosamines may occur in the environment and this has led to great improvements in the analytical methods. However, the amounts of carcinogenic nitrosamines detected and measured in food for human consumption are extremely small and their significance as a human health hazard is very difficult to assess. The formation of carcinogenic nitrosamines from amines and nitrites in the body has been demonstrated, and tumours have been induced by simultaneous administration of these materials to experimental animals. A number of drugs, pesticides and other environmental chemicals have secondary or tertiary amino structures and various amines occur in food. Nitrites are present in various vegetables and are added to certain foods as preservatives. Nitrates, which are found in drinking water, can be reduced to nitrites in the body and nitrites are present in human saliva. It is clear, therefore, that any amine in the stomach may be nitrosated, and perhaps give rise to a carcinogenic nitrosamine. Although the extent to which such reactions occur in human subjects under normal dietary conditions is not known, this possibility cannot be ignored (Magee, 1971, 1972, 1974).

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RECENT ADVANCES IN THE TOXICOLOGY OF N-NITROSO
AND HYDRAZINE COMPOUNDS

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Several N-nitroso compounds have found industrial use in chemical preparations and, in the case of dimethylnitrosamine, as a solvent. Many years ago it was recognized that these compounds can be toxic to man under factory and laboratory conditions (Watrous, 1947; Wrigley, 1948; Hamilton and Hardy, 1949; Barnes and Magee, 1954). In the early 1930's two young chemists involved in the manufacture of dimethylnitrosamine (DMN) became gravely ill after inhaling this compound (Freund, 1937). One man inhaled the vapors for over a month and became so ill he had to quit work; he suffered abdominal pain, nausea, and eventually ascites. After 18 months rest his condition was much improved yet he still had an enlarged tender liver. When he quit his job a second chemist carried on the work until one day he dropped a flask containing approximately a liter of DMN; he cleaned up the spill with a mop and rag and that night became very ill. Six days after the accident he developed pain in the upper abdomen and jaundice; in another 10 days ascites was pronounced. An exploratory laparotomy indicated a severely damaged liver and spleen. He died 48 days after the accident with severe hemorrhagic necrosis of the liver.

Dimethylnitrosamine was reported to have been responsible for the accidental poisoning of two men in an American automobile factory (Hamilton and Hardy, 1949). One worker developed signs of liver damage but recovered; an autopsy performed on the other victim who died in a clinical accident revealed a cirrhotic liver with regenerating nodules. In a British industrial research laboratory two of three men working with DMN for several months developed signs of liver injury (Barnes and Magee, 1954). One technician developed a hard liver with an irregular surface but recovered after exposure to the solvent was terminated. The other died of bronchopneumonia and necropsy revealed liver cirrhosis.

Certain nitroso compounds can be reduced to form hydrazine derivatives which are used as chemotherapeutics and rocket fuels. Hydrazine itself is a deoxygenator and is used in boiler water to prevent corrosion. Reid (1965) reported a case of an English sailor, while working in the ship's engine room, who

accidentally drank hydrazine instead of water. After vomiting, he collapsed and became unconscious. His cardiovascular, respiratory, and central nervous systems appeared normal when he regained consciousness, but he had an ataxia which persisted almost two weeks. In another case (Sotaniemi et al., 1971) a machinist handled hydrazine once a week for six months, each time experiencing nausea, tremor and conjunctivitis. He developed a cough, fever, diarrhea, vomiting, jaundice and stupor and died almost seven months after the first exposure. A histopathologic examination showed changes similar to those seen in experimental animals poisoned with hydrazine: severe tubular necrosis and nephritis and small foci of liver cell necrosis.

ACUTE TOXICITY

The acute toxicities of N-nitroso compounds have recently been reviewed by Magee (1971) and Shank (1974). The N-nitroso compounds can be divided into two chemical groups (Figure 1), the N-nitrosamines which require metabolic activation and the N-nitrosamides which, being unstable at physiologic pH, decompose to active intermediates presumably analogous to those derived from the nitrosamines.

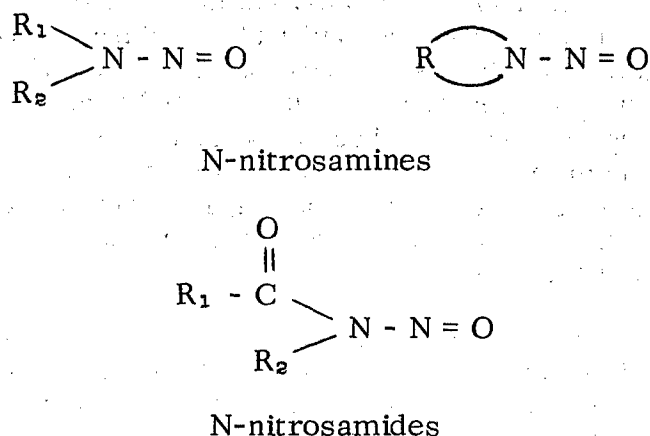


Figure 1. General structures for N-nitroso compounds.

The acute toxicities for these compounds vary greatly (Table 1); a clear structure-activity relationship is not yet apparent and much more work needs to be done in this area. It seems that acute toxicity decreases with chain length of dialkyl nitrosamines; substitution of one of the alkyl groups with an aromatic group may increase or decrease toxicity. Aliphatic ring N-nitroso compounds also can be acutely toxic.

TABLE 1. ACUTE TOXICITY OF SOME N-NITROSO COMPOUNDS

<u>Compound</u>	<u>LD₅₀*</u>	<u>Reference</u>
Dimethylnitrosamine	27-41	Heath and Magee, 1962
Diethylnitrosamine	216	Heath and Magee, 1962
Di-n-propylnitrosamine	> 400**	Pour et al., 1973
Di-n-butylnitrosamine	1200	Druckrey et al., 1964b
Di-n-amylnitrosamine	1750	Druckrey et al., 1961
Methyl-n-butylnitrosamine	130	Heath and Magee, 1962
Methyl-t-butylnitrosamine	700	Heath and Magee, 1962
Ethyl-n-butylnitrosamine	380	Druckrey et al., 1963a
Ethyl-t-butylnitrosamine	1600	Druckrey et al., 1963a
Ethyl-2-hydroxyethylnitrosamine	> 7500	Druckrey et al., 1963a
Di-2-hydroxyethylnitrosamine	> 5000	Schmahl, 1963
Methylphenylnitrosamine	200	Heath and Magee, 1962
Methylbenzylnitrosamine	18	Druckrey et al., 1963a
Nitrosomorpholine	282	Druckrey et al., 1961
Methylnitrosourea	180	Druckrey et al., 1961
Methylnitrosourethane	240	Druckrey et al., 1962a
Nitrosohexamethyleneimine	336	Goodall et al., 1968
Nitrosoheptamethyleneimine	283	Lijinsky et al., 1969
Nitrosooctamethyleneimine	566	Lijinsky et al., 1969

* LD₅₀ units: mg/kg body weight, single oral dose, adult male rats.

** Male and female Syrian golden hamsters.

The two simplest dialkylnitrosamines, DMN and diethylnitrosamine (DEN) have been used extensively in histopathologic studies on N-nitroso poisoning in experimental animals. Generally, they are hepatotoxins producing centrilobular hemorrhagic necrosis in 24 to 48 hours; the animals die in 3 to 4 days after single exposure or recover fully in a few weeks. It is unfortunate that the acute toxicities of the N-nitroso compounds have not been studied more systematically, but the striking carcinogenic properties of this class of compounds have diverted most attention toward chronic toxicity studies, short-cutting a firm understanding of the events which take place in an acute insult.

The simple hydrazines are also potent acute toxins (Table 2). LD₅₀ values for hydrazine, monomethylhydrazine (MMH), unsymmetrical 1,1-dimethylhydrazine (UDMH), and symmetrical 1,2-dimethylhydrazine (SDMH) are independent of route of administration in the rat, and except for hydrazine, this is also true in the mouse. This seems to indicate that these compounds are readily absorbed from the gut and peritoneum. Table 2 also demonstrates these compounds are hazardous as inhaled toxicants. It is interesting to note that MMH is more toxic to the rat, mouse and dog than the other indicated

hydrazines but is the only one for which there is no evidence of it inducing de novo neoplastic changes after chronic exposure. Indeed, an obvious relationship between acute toxicity and carcinogenesis for the hydrazines or nitroso compounds has not yet been recognized.

TABLE 2. ACUTE TOXICITIES OF SOME HYDRAZINES*

<u>Species</u>	<u>Route</u>	<u>HYD</u>	<u>MMH</u>	<u>UDMH</u>	<u>SDMH</u>
Rat	po	60 ^a	32 ^a	122 ^a	160 ^a
	ip	64 ^b	28 ^b	102 ^b	163 ^a
	iv	--	33 ^a	119 ^a	175 ^a
	ih	570 ^c	74 ^c	252 ^c	280 ^c
Mouse	po	59 ^a	33 ^a	265 ^a	36 ^a
	ip	163 ^d	32 ^a	290 ^a	35 ^a
	iv	57 ^a	33 ^a	250 ^a	29 ^a
	ih	252 ^c	56 ^c	172 ^c	--
Dog	iv	25 ^a	12 ^a	60 ^a	100 ^a

* LD₅₀ mg/kg or LC₅₀ ppm/4 hr.

HYD = hydrazine; MMH = monomethylhydrazine; UDMH = 1, 1-dimethylhydrazine; SDMH = 1, 2-dimethylhydrazine.

^a Witkin, 1956.

^b O'Brien et al., 1964.

^c Jacobsen et al., 1955.

^d Krop, 1954.

Hydrazine is a convulsant at high doses and may be a central nervous system depressant at low doses; animals that survive the immediate effects of hydrazine exposure may die a few days later of liver and kidney damage. Monomethylhydrazine in some species is a renal toxin and in others a hepatotoxin. Unsymmetrical dimethylhydrazine is a convulsant but doesn't induce liver damage. The toxicity of these compounds has recently been reviewed by Clark et al. (1969) and Back and Thomas (1970).

Magee and Barnes (1956) were the first to demonstrate the carcinogenicity of an N-nitroso compound, and since then most, but not all, available N-nitroso compounds have been shown to be carcinogenic. As a chemical group, these agents have some remarkable properties for tumor production. They are capable of producing tumors in a great many tissues (Table 3); a single administration given to neonatal animals can produce a high tumor incidence upon reaching adulthood (Magee and Barnes, 1959; Druckrey et al., 1964c).

TABLE 3. TUMOR SITES OF SOME N-NITROSO COMPOUNDS

<u>Site</u>	<u>Compound</u>	<u>Reference</u>
Skin	Methylnitrosourea	Graffi & Hoffmann, 1966
Nose	Diethylnitrosamine	Herrold, 1964
Nasal sinus	Dimethylnitrosamine	Druckrey et al., 1964a
Tongue	Nitrosohexamethyleneimine	Goodall et al., 1968
Esophagus	Nitrosoheptamethyleneimine	Lijinsky et al., 1969
Stomach	Ethylbutylnitrosamine	Schmahl et al., 1963
Duodenum	Methylnitrosourea	Druckrey et al., 1963b
Colon	Cycasin	Laqueur, 1965
Lung	Diethylnitrosamine	Dontenwill & Mohr, 1961
Bronchi	Diethylnitrosamine	Dontenwill & Mohr, 1961
Liver	Dimethylnitrosamine	Magee & Barnes, 1956
Pancreas	Nitrosomethylurethane	Druckrey et al., 1968
Kidney	Dimethylnitrosamine	Magee & Barnes, 1959
Bladder, u.	Dibutylnitrosamine	Druckrey et al., 1962b
Brain	Methylnitrosourea	Druckrey et al., 1965
Spinal cord	Nitrosotrimethylurea	Ivankovic et al., 1965
Thymus	Nitrosobutylurea	Yokoro et al., 1970
Lymph nodes	Ethylnitrosourea	Vesselinovitch et al., 1971
Blood vessels	Nitrosomorpholine	Bannasch & Mueller, 1964 Newberne & Shank, 1973

Even in adult animals a single administration can produce tumors; malignant genital tumors result from a single administration of N-nitrosoethylurea to pregnant rats (Druckrey and Ivankovic, 1969); a single dose of DMN to adult rats can induce kidney tumors (Magee and Barnes, 1959) and even liver tumors if the rat had been partially hepatectomized when dosed (Craddock, 1973).

The N-nitroso compounds can induce tumors transplacentally. N-nitrosoethylurea given on the 15th day of gestation produces tumors of the brain and spinal cord of the offspring (Ivankovic and Druckrey, 1968);

the same compound has also produced kidney tumors transplacentally (Wrba et al., 1967). Diethylnitrosamine given to rats on the 9th through 15th day of gestation induced tracheal papillomas in the offspring (Mohr et al., 1966).

Activation of the N-nitroso compounds seems to be necessary for tumor production. Dimethylnitrosamine is oxidized more rapidly in rat liver than in kidney and chronic, low-level exposure of rats to DMN produces more liver tumors than kidney tumors. Rat lung is a poor site for DMN metabolism and rarely are lung tumors produced (Magee and Barnes, 1967). On the other hand, diethylnitrosamine is more readily metabolized by hamster lung than liver and it induces in hamsters more lung than liver tumors (Montesano and Magee, 1971). Nitrosamides often produce tumors at the site of administration, apparently because they break down spontaneously under physiological conditions and therefore don't require metabolic activation.

This species and tissue specificity for the nitrosamines may be of value in extrapolating from animal data to man. Obviously one cannot screen potential carcinogens in man, but since the correlation between metabolic activation and tumor production is high, animals can be used to identify the active metabolite and then human tissue can be examined in vitro to determine its capacity to produce the active metabolite and presumably to become cancerous. DNM has produced tumors in every species that metabolized it and recently, Montesano and Magee (1971) have reported that human liver can metabolize DMN; thus the prediction that man is probably susceptible to the carcinogenic potential of DMN does not seem to be incautious.

The carcinogenicity of the hydrazines is less well defined. Unfortunately, much of the screening work has been done in the mouse in which spontaneous tumor rates are high, and increases in these rates or reduction in induction time are interpreted as evidence for carcinogenicity, that is, de novo malignant tumor formation. Hydrazine and hydrazine sulfate increase the incidences of lung adenomas and carcinomas and hepatomas (Biancifiore and Ribacchi, 1962; Biancifiore et al., 1964; Biancifiore, 1970a and 1970b). Reticulum-cell sarcomas have been obtained in mice in which the spontaneous rate is low (Juhasz et al., 1966).

Toth (1972a) has shown that monomethylhydrazine shortens the latent period for pulmonary tumors in mice, and Roe et al. (1967) observed increases in the incidences of pulmonary tumors in female Swiss mice treated with hydrazine or 1,1-dimethylhydrazine. Stronger evidence supports the carcinogenicity of symmetrical 1,2-dimethylhydrazine. Osswald and Kruger (1969) obtained hepatocarcinomas and adenocarcinomas of the gastrointestinal tract in hamsters given symmetrical dimethylhydrazine. Schauer et al. (1969) and Springer et al. (1970) found intestinal adenocarcinomas, especially in the ascending and transverse colon, in rats treated with 1,2-dimethylhydrazine. In hamsters the compound produces angiosarcomas of the vascular system and cecal and liver tumors (Toth, 1972b). Little is known about the relationship between the metabolism of the hydrazines and their carcinogenicity.

METABOLISM OF THE N-NITROSO COMPOUNDS AND HYDRAZINES

The metabolism of the nitrosamines, especially DMN, has been the object of almost 20 years of study and the biologically active metabolite is not yet known; indeed, it is likely that all the metabolic pathways for DMN have not been described.

Magee (1956) and Dutton and Heath (1956) demonstrated that the liver is capable of metabolizing DMN, oxidizing the methyl group(s) to carbon dioxide. Magee and Vandekar (1958) showed that the disappearance of DMN by rat liver slices and rabbit liver microsomes required NADPH and molecular oxygen. Rose (1958) suggested that the active molecule in DMN toxicity and carcinogenicity might be diazomethane. This was supported by the finding of an N-demethylase for DMN in rat liver and that DMN metabolism led to methylation of rat liver proteins (Magee and Hultin, 1962) and nucleic acids (Magee and Farber, 1962).

After the enzymatic oxidation of DMN, the methyl-hydroxymethylnitrosamine is thought to breakdown spontaneously to monomethylnitrosamine and formaldehyde (Figure 2); the amount of formaldehyde produced is insufficient to account for the toxicity of DMN. The monomethylnitrosamine undergoes a protonic change to yield the diazohydroxide (Druckrey et al., 1961a); these products are unstable and decompose in a fraction of a second (Muller et al., 1960). It is not known how the diazohydroxide breaks down but it has been suggested (Rose, 1958; Druckrey et al., 1961a) that diazomethane, methyldiazonium ion or the diazotate could be intermediates. Lijinsky and co-workers (1968) however, have shown that DMN alkylation of rat liver DNA and RNA is a transmethylation with no exchange of hydrogen atoms involved in deriving the methonium ion, thus arguing strongly against the formation of diazomethane in DMN metabolism.

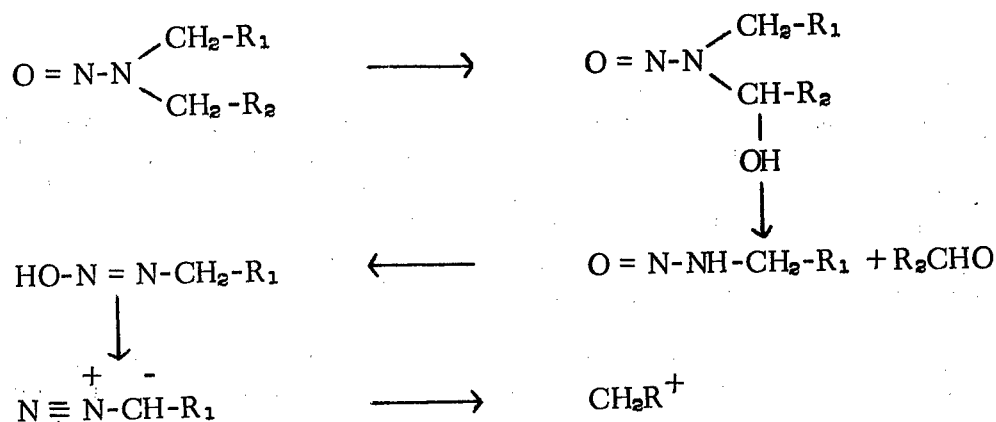


Figure 2. Metabolism of N-nitrosamines via alpha-hydroxylation; when $\text{R}_1 = \text{R}_2 = \text{H}$, the parent compound is dimethylnitrosamine.

To account for metabolism of cyclic and higher dialkyl nitrosamine, Druger (1972) proposed a metabolic pathway similar to the β -oxidation of fatty acids (Figure 3). Enzymatic dehydrogenation between the α and β carbons of one alkyl chain and addition of water to the resultant double bond produces a β -hydroxylated nitrosamine because of the inductive effect of the nitroso group, analogous to the effect that activated carbonyl group has in β -oxidation of fatty acids. The β -hydroxylated nitrosamine is further oxidized, yielding acetyl CoA and methylalkyl nitrosamine. The nitrosamine apparently then undergoes α -oxidation, as above, producing either the methonium ion or the carbonium ion corresponding to the second alkyl group.

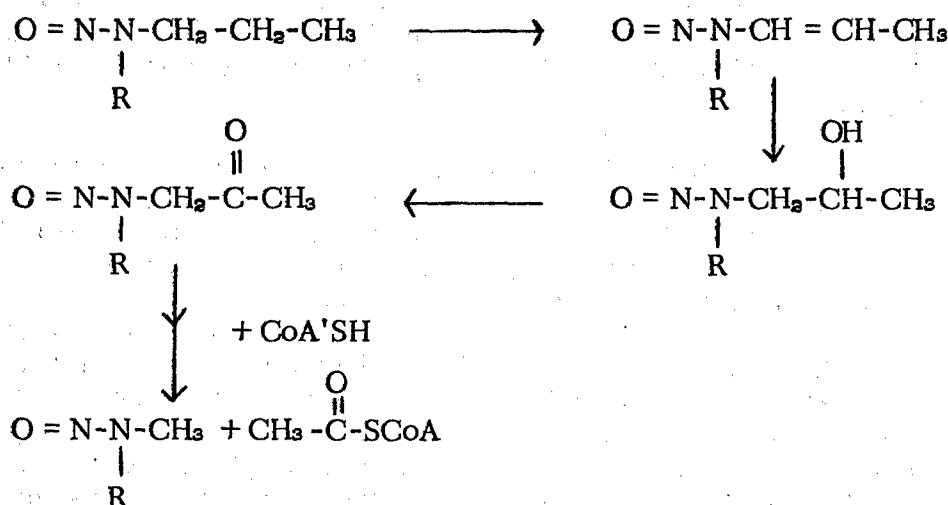


Figure 3. Metabolism of N-nitrosamines via beta-hydroxylation; the example illustrated is alkylpropyl nitrosamine.

Using alkylation of liver RNA guanine as evidence for the metabolic formation of carbonium ions, Kruger (1972) found that dipropyl nitrosamine-1- ^{14}C metabolism yielded both 7-methyl- ^{14}C -guanine and 7-propyl- ^{14}C -guanine in the RNA. When the 2-position of each propyl chain was labelled, only 7-propylguanine in RNA was labelled. Also, as predicted by the proposed β -oxidation pathway, metabolism of dibutyl nitrosamine-1- ^{14}C yielded both 7-methyl- ^{14}C -guanine and 7-butyl- ^{14}C -guanine.

Metabolic intermediates of dipropyl nitrosamine predicted by the β -oxidation hypothesis did produce the expected alkylated bases and thus presumably produced the anticipated carbonium ions (Kruger and Bertram, 1973; Althoff et al., 1974); however, when these same intermediates were given to hamsters weekly for life they produced tumors in tissues not targets

for the parent nitrosamine (Pour et al., 1974a and 1974b). Further studies are in progress to clarify these observations, but the majority of evidence supports the existence of both α and β oxidation for nitrosamines.

Almost half of subcutaneously injected hydrazine is excreted unchanged in urine of rats and mice (Dambrauskas and Cornish, 1964). Grebennik (1967) claims that hydrazine can be excreted in urine as the free, bound, or acetylated compound in rats and rabbits but only as free hydrazine in dogs. Salvatore and co-workers (1955) have reported that rat liver, brain, kidney and muscle can enzymatically convert hydrazine to ammonia.

Far less is known of the metabolism of methylhydrazines. Wittkop and co-workers (1969) demonstrated that monomethylhydrazine (MMH), 1,1-dimethylhydrazine (UDMH), and 1,2-dimethylhydrazine (SDMH) are oxidatively demethylated to yield formaldehyde by rat liver microsomal preparations requiring oxygen, NADPH and cytochrome P-450. This enzyme system is similar to the mixed function oxidases responsible for the hydroxylation of many foreign compounds.

Cycasin is neither a nitrosamine nor a hydrazine but a naturally-occurring azoxy- β -glucoside (Nishida et al., 1955) which has indirectly been associated with human illness (not cancer) in Guam (Whiting, 1963). The glucoside is not toxic but is hydrolyzed by gut flora to methylazoxymethanol (man), which is highly toxic and carcinogenic (Spatz et al., 1967). This compound is proving valuable to the understanding of how the nitrosamines and hydrazines are metabolically transformed to active molecules.

Progeny of pregnant rats treated with azoxymethane or 1,2-dimethylhydrazine do not develop tumors; but MAM is carcinogenic to progeny of treated dams. This supports Druckrey's suggestion that oxidation of a carbon atom to form an alkyl diazohydroxide or alkyl azoalcohol is critical to the activation process. There is also support for this in that administration of dimethylnitrosamine (Magee and Farber, 1962), 1,2-dimethylhydrazine (Hawks et al., 1972), or methylazoxymethanol (Shank and Magee, 1967) leads to alkylation of nucleic acids, presumably by yielding the same alkylating agent. These interrelationships are shown in Figure 4.

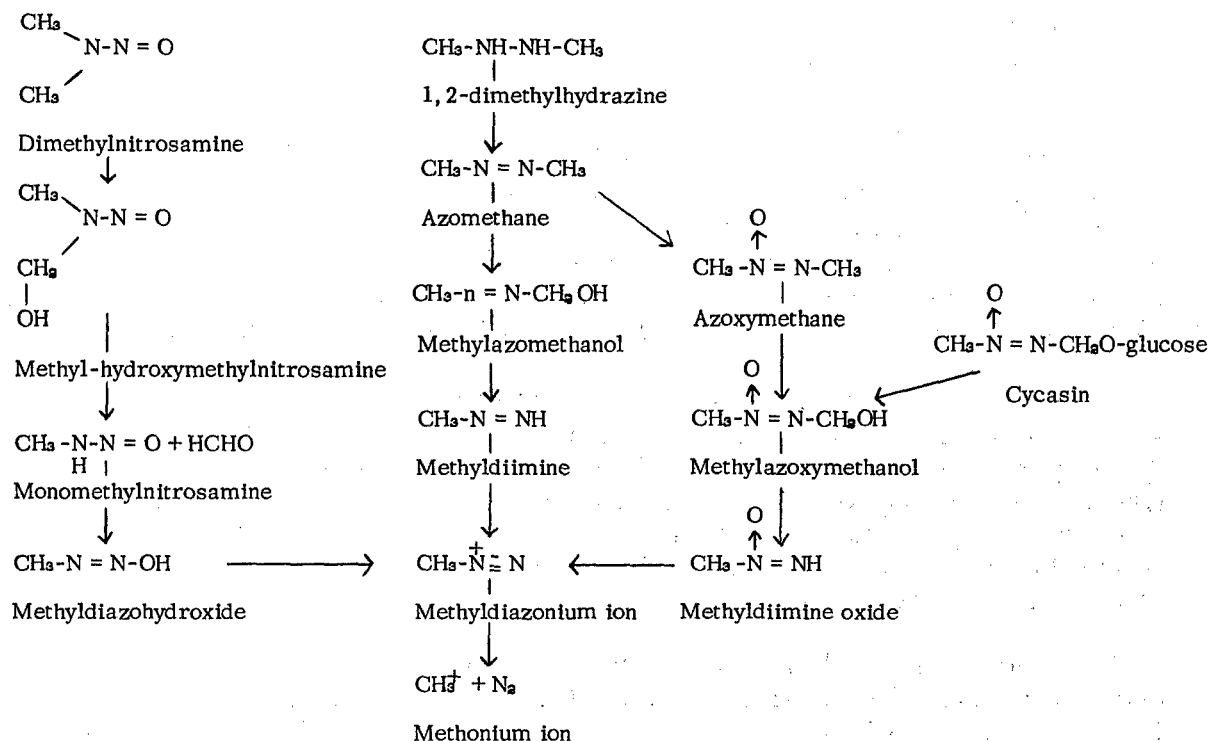


Figure 4. Relationships between the metabolism of dimethylnitrosamine, 1,2- dimethylhydrazine and cycasin.

Intense interest has focused on the generation of this active alkylating agent, for it is thought that this carbonium ion produces both lethal alterations in the genetic codes of RNA and DNA resulting in death of the cell and non-lethal transcribable changes resulting in transformation to a cancerous cell. Alkylation of the 7-position of guanine seems to be closely related to acute toxicity in that production of 7-methyl-guanine in messenger RNA inactivates guanine in the genetic code (Wilhelm and Ludlum, 1966), effectively inhibiting translation in protein synthesis (Villa-Trevino, 1967; Shank, 1968); however, polyribonucleotides containing 7-methyl-guanylic acid serve as normal templates for RNA polymerase (Ludlum, 1970). The induction of transcribable alterations in the genetic code resulting in transformation to a cancerous cell is an extremely complex problem and will be discussed in the next paper.

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THE EFFECTS OF 6-MONTH CHRONIC LOW LEVEL INHALATION
EXPOSURES TO HYDRAZINE ON ANIMALS

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Hydrazine (N_2H_4) is a highly reactive reducing agent which is widely used as an intermediate in organic synthesis and either singly or in combination with other hydrazines such as 1,1-dimethylhydrazine or methylhydrazine as a missile propellant. It is also used extensively as a corrosion inhibitor in boiler feed water. Hydrazine is a colorless liquid with a molecular weight of 32.05, density of 1.008 g/ml and a vapor pressure of 14.4 mm Hg at 25 C.

Hydrazine is a strong convulsant at high doses but may cause central nervous system depression at lower doses. Its toxicity and pharmacologic effects are detailed in a comprehensive review by Clark et al. (1968). Animals may die acutely of convulsions, respiratory arrest, or cardiovascular collapse within a few hours of an acute exposure by any route of administration, or may die 2 to 4 days later of liver and kidney toxicity (Weir et al., 1964; Witkin, 1956). Jacobson et al. (1955) reported the 4-hour inhalation LC_{50} value as 252 ppm (330 mg/m³) for the mouse and 570 ppm (750 mg/m³) for the rat.

House (1964) exposed monkeys, rats and mice to a hydrazine concentration of 1.0 ppm continuously for 90 days. Though mortality was very high, some animals survived the experiment. Ninety-six percent of the rats and 98% of the mice died during the exposure while monkeys proved to be the most resistant species with only a 20% mortality.

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Comstock et al. (1954) exposed dogs, in separate experiments, to 5 and 14 ppm. Two dogs survived repeated 6-hour exposures to 5 ppm hydrazine for 6 months and 2 of 4 dogs lived after 194 six-hour exposures to 14 ppm; the other two dogs died during the third and fifteenth weeks in a debilitated condition. The dog that died during the fifteenth week had a severe convulsive seizure prior to death. Prior to death, both dogs showed signs of anorexia and general fatigue. Changing diets and forced feedings resulted in the survival of the remaining two dogs.

The present Threshold Limit Value (TLV) published by the American Conference of Governmental Industrial Hygienists (1973) for N_2H_4 is 1 ppm or 1.3 mg/m³.

To compare the effects of repeated 6 hour per day, 5 day per week (industrial type) exposures with continuous exposures of equivalent concentrations and to evaluate the safety factor of the current TLV, four concentration levels were selected for the 26-week exposure of four animal species. The concentrations selected were: 1.0 ppm and 0.2 ppm for continuous exposures and 5.0 ppm and 1.0 ppm for intermittent daily exposures. These concentrations would result in the following CT (concentration x time) values:

1.0 ppm continuous	=	168 ppm-hours per week
5.0 ppm intermittent	=	150 ppm-hours per week
1.0 ppm intermittent	=	30 ppm-hours per week
0.2 ppm continuous	=	33.6 ppm-hours per week.

Thus, the 1.0 ppm continuous and the 5.0 ppm intermittent studies would be relatively equivalent doses and the 1.0 ppm intermittent and 0.2 ppm continuous would also be comparable.

Four exposed groups and a control group were used in these experiments. Each consisted initially of 8 male beagle dogs, 4 female rhesus monkeys, 50 male Sprague-Dawley rats and 40 female CF-1 mice. The animals were monitored throughout the 6 months of exposure with biological measurements made at biweekly intervals. These measurements consisted of hematology and clinical chemistry values, body weights, physical examinations, and on selected animals bone marrow examinations were conducted. The details of the experimental methodology and findings were presented by Haun and Kinkead (1973) with the exception of the results in groups of rats and mice held for long-term postexposure observation. Ten rats and 10 mice from each experimental and control group were set aside at the end of the 6-month exposure period and maintained in an animal holding room.

The effects of chronic inhalation of hydrazine are dose related regardless of the nature of exposure, i.e., intermittent or continuous. The highest hydrazine dose caused approximately 40% deaths in mice within the first two months of exposure while the TLV dose equivalents caused approximately 5% mortality.

Although mice were not weighed, rats exhibited a dose related growth rate depression and dogs exposed to hydrazine showed weight loss at the highest dose levels.

The most significant or noticeable signs of stress occurred in the case of the dogs exposed to 1 ppm continuously. Weight loss was very noticeable in these dogs, and although we did not measure food consumption, it was obviously reduced. Anorexia continued with progressive emaciation until about 16 weeks when some recovery occurred in the surviving dogs. One dog in this group experienced tonic convulsions on 3 separate occasions, once after 3 months of exposure, then once in the morning and once in the afternoon of the same day after 5 months of exposure. These findings were consistent with those reported by Comstock et al. (1954).

In animals held postexposure, weight differences between control and exposure groups became insignificant by four weeks.

There were no abnormal findings in clinical chemistry and hematology measurements made on monkeys and rats. Dogs, however, had a hydrazine dose related depression of red blood cell counts, hemoglobin values, hematocrits, and there was little or no reticulocytosis before the fifth month of exposure at which time the dogs continuously exposed to 1 ppm N_2H_4 had a sharp depression of RBC count accompanied by reticulocytosis. At necropsy, this group of dogs was the only group of any species to demonstrate erythropoietic activity as measured by a decreased myeloid/erythroid ratio in bone marrow.

There was no measurable evidence of red blood cell destruction in these dogs exposed to hydrazine in contrast with readily demonstrated hemolytic activity of monomethylhydrazine (MMH) (MacEwen and Haun, 1971). Furthermore, the red blood cells of dogs exposed to N_2H_4 were markedly more susceptible to osmotic fragility than control animals while MMH produced a significant increase in RBC fragility. We were unable to determine the precise reason for the hydrazine induced anemia or to explain the decreased RBC fragility during these experiments but plan to explore this area further.

The results of gross and histopathologic examination of mice that died during exposure showed that death was due to hydrazine hepatotoxicity. At sacrifice, moderate to severe fatty liver change was a consistent finding in mice from all exposure levels. Monkey livers showed slight to moderate fat accumulation. Perhaps compromising part of this information is the fact that control monkeys also showed some degree of fatty liver change. Malnutrition, the result of nonspecific hydrazine toxicity, caused the death of 2 dogs in the 1 ppm continuous exposure. At sacrifice, dogs exposed to the TLV concentration showed no abnormalities but dogs from the high doses had fatty livers. Since one dog in the 1 ppm continuous exposure group convulsed during exposure, the brains of this dog and 3 others in the same group were perfused at

sacrifice. Histology revealed no CNS lesions. Two dogs each from the high concentration experiments were sacrificed at 6 weeks postexposure. All were described as being essentially normal animals.

Organ weights of exposed rats, monkeys and dogs were not statistically different from control values. In the case of the rats, the depressed growth rates resulted in increased organ to body weight ratios to which no biological significance can be attributed.

There were no significant pathologic changes in rats except in the case of the 5 ppm intermittent exposure group. Of the 30 rats, 19 had chronic bronchopneumonia. Whether this condition was due to a hydrazine pulmonary irritation or pathogens present, or the former predisposing the rats to the latter, is difficult to say. The net effect, however, was that 10 rats from this group retained postexposure showed no weight recovery as demonstrated by the other exposed groups. The infection spread to other rat groups housed in the same room and within 6-8 weeks following exposure termination, 50% of the rats were dead. The number of deaths was distributed rather evenly in the exposed groups and in the controls as well. Consequently, none of the rats survived long enough for conclusions to be drawn about the carcinogenic potential of inhaled hydrazine for this species.

Tumorigenesis has, however, been demonstrated in rats following daily oral administration of 12 or 18 mg hydrazine sulfate doses over a 68 week period. Pulmonary adenocarcinomas, hepatic cell and spindle cell carcinomas were observed after 109 weeks (Severi and Biancifiori, 1968). They found no lung or liver tumors in their untreated control rats.

Approximately half the mice in each group were alive 1 year postexposure. At necropsy, non-neoplastic lesions were found in the ICR/CF-1 mice with approximately equal frequency in both experimental and control groups. An occasional mouse had a mammary gland adenoma, but since these are normally found with an incidence of 5-10% in mice, they were considered to be unrelated to the hydrazine exposure. For similar reasons, a single small papilloma found in one exposed mouse was not considered significant.

Some of the mice exposed to the threshold limit value concentration of hydrazine (1 ppm) had well differentiated alveolargenic carcinomas as shown in Figure 1. Another of these tumors (Figure 2) shows invasion of the pleura and extension into the pleural space. In mice of the 5 ppm intermittent exposure group, alveolargenic carcinomas were also seen. These tumors had a greater frequency of metastatic activity with tumors found in liver and in the ribs as shown in Figures 3 and 4. Many mice exposed to 1 ppm hydrazine on a continuous basis developed alveolargenic carcinomas. One had a hepatoma as shown in Figure 5. There was one rather poorly circumscribed area of the liver in which the cells were large with variable sized nuclei, many of

which contained a large eosinophilic intranuclear inclusion. In this same animal most of the spleen was replaced by neoplastic tissue (Figure 6). Most of this tissue was very anaplastic as shown in Figure 7, but in a few areas the cells resembled neoplastic hepatocytes. Two mice in this group developed lymphosarcoma in the spleen (Figure 8) which was extremely cellular with a loss of normal architecture. The cells are uniformly immature lymphocytes with numerous mitotic figures. There is invasion of the capsule with a great deal of phagocytosis by macrophages. This, in combination with the other morphology, is diagnostic of lymphosarcoma and is substantiated by an adjacent lymph node (Figure 9) which shows complete loss of architecture due to these neoplastic lymphoid cells. Similar changes were also seen in liver, kidney, lung and the urinary bladder.

The tumor incidences shown in Table 1 are believed significant for two reasons. First, alveolargenic carcinomas are found in higher, dose related, frequencies among exposed mice than in controls. Second, lymphosarcoma and the uncommon malignant hepatoma are absent from controls but occur in mice exposed to the higher dose.

TABLE 1. TUMOR INCIDENCE IN MICE ONE YEAR AFTER
CHRONIC INHALATION EXPOSURE TO HYDRAZINE
(6-MONTH EXPOSURE PERIOD)

<u>Exposure</u>	<u>Alveolargenic Carcinoma</u>	<u>Lympho- sarcoma</u>	<u>Hepatoma</u>	<u>Number of Mice with Tumors</u>
<u>High Dose</u>				
1.0 ppm Continuous	5/9	2/9	1/9	6/9
5.0 ppm Intermittent	5/6	0/6	0/6	5/6
<u>Low Dose</u>				
0.2 ppm Continuous	3/8	0/8	0/8	3/8
1.0 ppm Intermittent*	2/6	0/6	0/6	2/6
Control Group	1/8	0/8	0/8	1/8

*Current Threshold Limit Value (TLV)



Figure 1. Typical alveolargenic carcinoma found in mouse lung - 8X (1278-74).

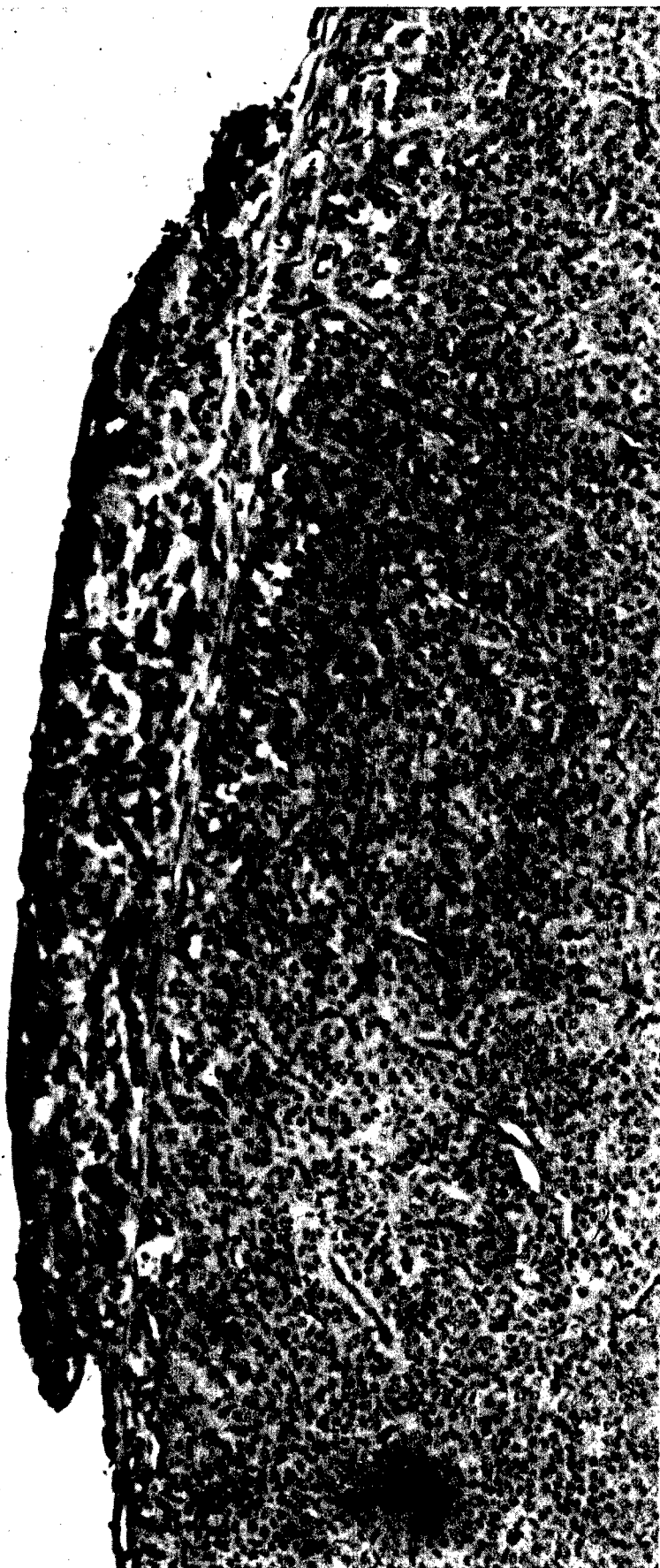


Figure 2. Invasion of pleura by alveolar carcinoma - 8X (1276-74).



Figure 3. Metastatic lesion of mouse alveolar carcinoma in liver - 33X
(1271-74).



Figure 4. Metastatic lesion of mouse alveolar carcinoma in intercostal muscle - 8X (1271-74).

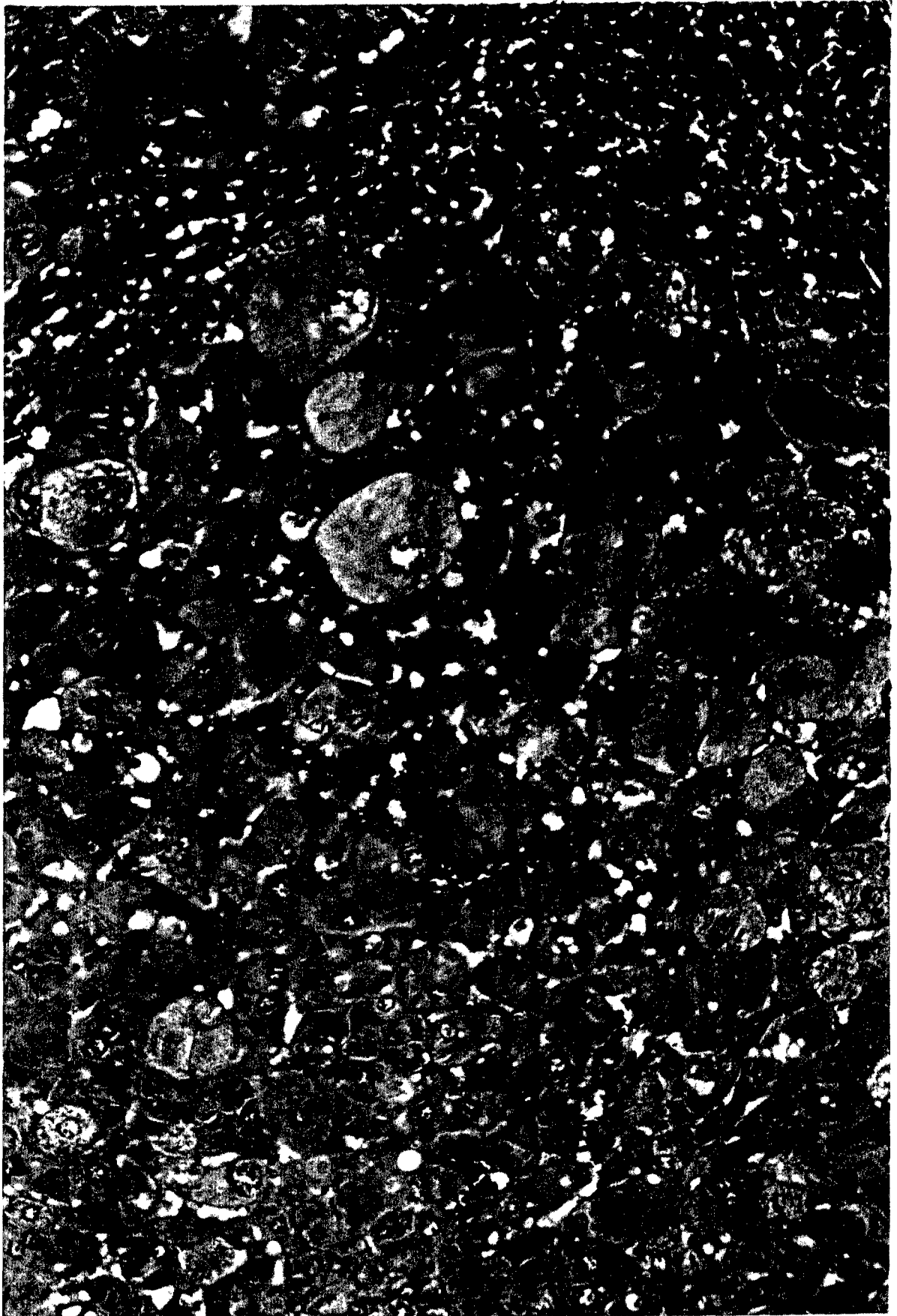


Figure 5. Unorganized liver tissue in mouse hepatoma - 33X (1267-74).



Figure 6. Splenic hyperplasia in mouse metastatic hepatoma - (1267-74).

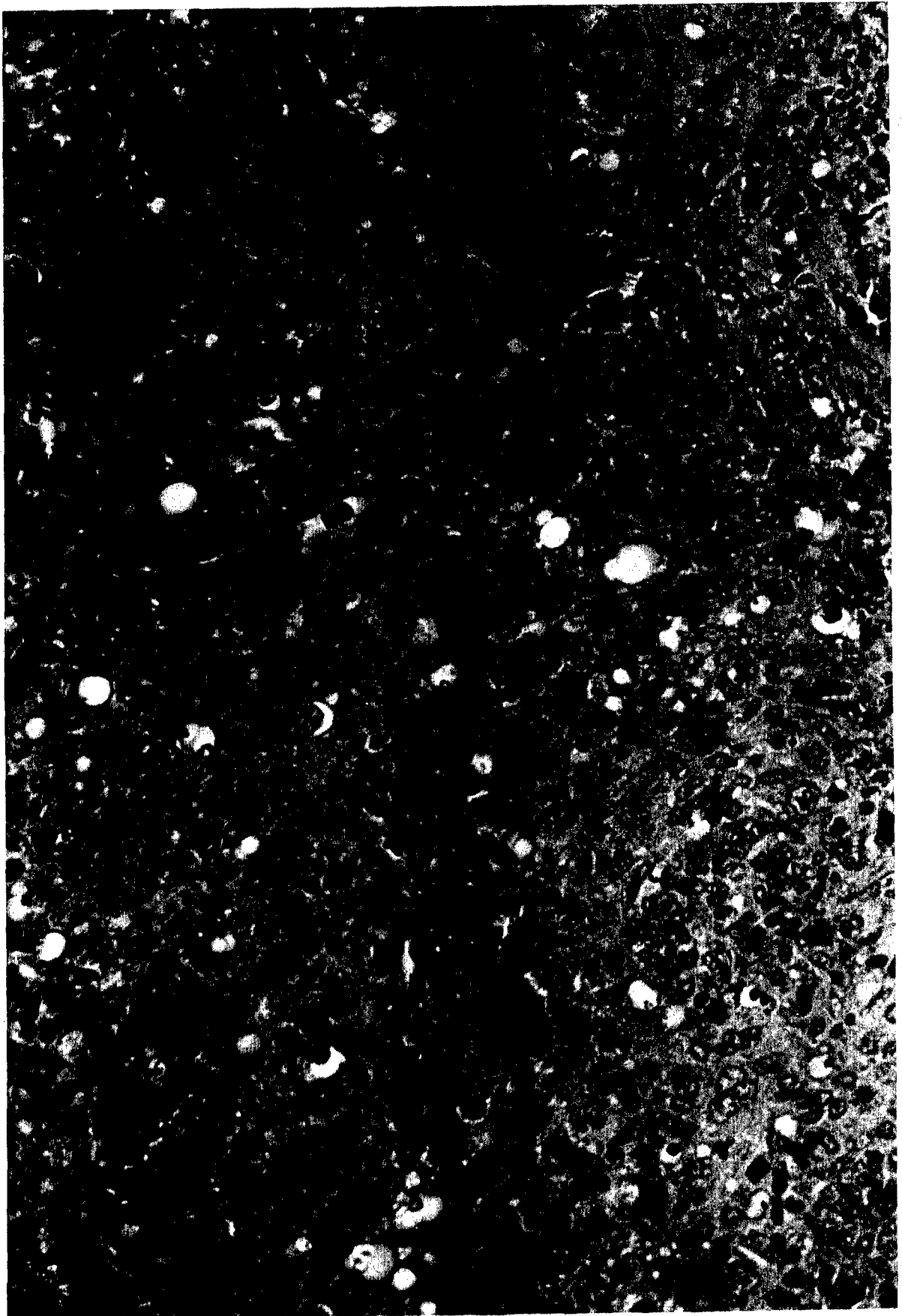


Figure 7. Splenic neoplasia in mouse hepatoma - 65X (1267-74).



Figure 8. Splenic changes seen with lymphosarcoma in the mouse - 33 X
(1265-74).



Figure 9. Neoplastic changes seen in a lymph node with lymphosarcoma - 8X
(1265-74).

Alveolargenic carcinomas are "normally" found in older mice with a frequency of about 10% and we have found this rate of tumors in this experiment as well as in other concurrent experiments in this laboratory using the CF-1 strain mice. In previous studies, using electron microscopy, these tumors have been shown to contain Type C virus particles. The virus particle is thought to be the probable etiologic agent for these spontaneous alveolar carcinomas. Another significant finding is the metastatic extension of the hepatoma to the spleen and metastasis of alveolargenic carcinomas to heart and rib cage, respectively, in two other hydrazine exposed mice. These findings are consistent with the induction of lung tumors in mice by hydrazine sulfate reported by Biancificori et al. (1962a, 1962b, 1963a, 1963b, 1963c, 1966), Biancificori (1969 and 1970), Roe et al. (1967), and Toth (1969, 1971, 1972). Hepatomas and hepatocarcinomas have also been observed after oral dosing of mice with hydrazine sulfate by Biancificori (1970a, 1970b, 1970c, 1971) and by Biancificori et al. (1964). The significance of the findings reported is that this is the first demonstration of hydrazine induced tumors from simulated industrial inhalation exposures at the TLV concentration in mice, albeit in small numbers of animals. These findings should be expanded in additional experiments exposing large numbers of animals of several species to hydrazine by the inhalation route.

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EVALUATION OF EXPERIMENTAL DATA FOR ASSESSING
CARCINOGENIC RISK FOR MAN

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INTRODUCTION

It is our intent in this paper to examine the track record of toxicology in predicting chemically induced cancer in man. Our purpose in so doing is to identify what lessons might be learned that would lead to improved predictions and, perhaps, to greater applications of carcinogenicity testing.

We do not pretend to be the first or the best to undertake such a study. We certainly hope we are not the last since we believe this should be a regular, iterative process.

We intend to review the data from animal experimentation and human experience for several well-known carcinogenic compounds. The extent of the match between the two sets of data will be noted. Where the match is poor or non-existent, we will ask: Why? Where the match is good, we will ask: Was the prediction applied to the task of protecting human health? This, of course, is the ultimate purpose of toxicology.

The compounds chosen for this study are ones upon which there are extensive studies and which also are of current interest. Time does not permit our going into the entire list of hundreds of materials that have been evaluated for their carcinogenic potential.

It is generally accepted today that the majority of human cancers are caused by environmental factors (Lisella et al., 1972), but we are only beginning to identify those factors and to understand their mechanism of action.

In the best of all worlds, proper testing in laboratory animals would enable the prediction of those agents responsible for human cancer, their effect on man, and the dose range producing that effect.

Practically, animal testing procedures have often failed to enable man to predict - before the damage is done - those agents responsible for human cancers, what tumors to look for, and what doses are capable of inducing cancer in man.

Why do experimental data fall short of the ideal? Is it because of man's failure to take note? By way of introduction, let us look quickly at two examples. Early studies by Torkelson et al. (1961) and Mastromatteo et al. (1960) showed hepatic and renal damage following sub-chronic exposures to vinyl chloride. In a now well-known report, Viola et al. (1971) described tumor production by this agent in rats after chronic exposure. Three years elapsed before human hepatic angiosarcoma was causally related to vinyl chloride exposure. Viola's experimental design incorporated exposure of rats for one year to a concentration of 30,000 ppm of the carcinogen, a concentration man would not expect to be exposed to. Hence the results were not viewed with alarm. Lower concentrations were not tested - were they therefore presumed safe?

Diethylstilbestrol (DES), when taken by women during early pregnancy, has been implicated as the cause of particularly tragic cancers - vaginal carcinoma at puberty in the offspring (Herbst et al., 1971; Greenwald et al., 1971). Yet, as early as 1963, Dunn and Green reported animal studies which should have provided a warning - they found cervical carcinoma following estrogen injection in newborn mice. Even before that, investigations in 1957 indicated endometrial carcinoma in adult rabbits following diethylstilbestrol injection (Meissner et al., 1957). In this case, even the warning was too late to prevent the cancers, but it might have been in time to prevent some of the tragedies.

There may well be, probably are, many other similar instances - unfortunately - where hindsight is 20/20 and foresight at best is 20/400. One cannot help wondering if psychological factors are at work - refusal to see the obvious because of the seriousness of the consequences. An excellent example is the social history of tobacco smoking. In other cases, it may be that we refuse, for economic reasons, to withhold human exposure until adequate toxicity tests are completed: vinyl chloride is vital to the plastics industry; DES is vital to the cattle industry.

Do experimental data fall short of the ideal because tumors noted in laboratory animals have so frequently failed to be seen in man? Let's examine some of the data.

The literature on the 14 carcinogens listed by OSHA was reviewed. In seven of those 14 (Figure 1), there is no evidence that cancers have developed in man as a result of exposure to the compounds (2-AAF, 3,3'-dichlorobenzidine, dimethylaminoazobenzene, N-nitrosodimethylamine, 4,4'-methylene(Bis)2-chloroaniline, ethyleneimine, β -propiolactone). We have focused our attention on the other seven as shown in Figure 1. The human carcinogenic activity of α -naphthylamine and chloromethyl methyl ether is questionable. Epidemiologic evidence points to benzidine, β -naphthylamine, 4-aminodiphenyl, 4-nitrobiphenyl, and bis(chloromethyl) ether as acting to cause cancer in man. Of course, there are others known - perhaps 15 more - not listed by OSHA. But there are 1000 or more compounds known to cause cancer in laboratory animals (Delaney, 1972). What will be OSHA's next carcinogen?

KNOWN HUMAN CARCINOGENS LISTED BY OSHA

Figure 1

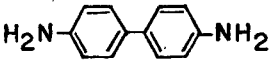
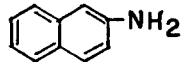
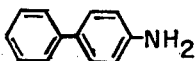
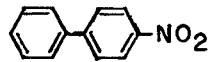
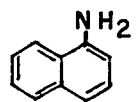
COMPOUND	STRUCTURE	ACRONYM
Benzidine		
β -Naphthylamine		β -NA
4-Aminodiphenyl		4-ADP
4-Nitrobiphenyl		4-NBP
Bis(chloromethyl) ether	$\text{ClH}_2\text{C}-\text{O}-\text{CH}_2\text{Cl}$	BCME
α -Naphthylamine		α -NA
Chloromethyl methyl ether	$\text{H}_3\text{C}-\text{O}-\text{CH}_2\text{Cl}$	CMME

Figure 1. Known human carcinogens listed by OSHA.

Included in those not on the OSHA list are the polycyclic aromatic hydrocarbons such as benz(a)anthracene, benzo(a)pyrene, chrysene, dibenz(a,h)anthracene, and dibenzopyrenes which have been tested extensively in the laboratory. Benz(a)anthracene is carcinogenic to the mouse by oral, subcutaneous and dermal application, and bladder implantation. Benzo(a)pyrene has proven carcinogenic in nine species, by routes as varied as oral, skin painting, inhalation, intratracheal, intraperitoneal, and subcutaneous injection, etc. Chrysene produces skin tumors in mice following skin painting. Dibenz(a,h)anthracene is carcinogenic in at least six species, by various routes, producing both local and systemic carcinogenic effects. The dibenzopyrenes have produced skin tumors in mice and local injection sarcomas in mice and hamsters (IARC, 1973b).

However, there is neither epidemiologic evidence, nor case reports, of a carcinogenic action of any of these compounds in man. It is important to note that they are found in the environment, and some are found in coal-tar, a known human carcinogen (IARC, 1973b).

Do experimental data fall short of the ideal because tumors seen in man fail to develop in laboratory animals? Again, consider the data.

Exposure to arsenic and some organic compounds of arsenic appears positively correlated with excess pulmonary (NIOSH, 1973; IARC, 1973a) and skin cancers (Tseng, 1968; Yeh, 1973; NIOSH, 1973; IARC, 1973a) in man. However, there is, as yet, no equally strong evidence that laboratory animals develop cancers when exposed to these compounds (IARC, 1973a). How many other "false-negatives" there are in the human environment, of course, no one can say. But if it is true that most human cancers are environmentally related, it seems reasonable to suppose that there are many other false negatives, or that we are not yet testing the right compounds in the right way, or that we are not looking at the data in the proper light. Of the thousands and thousands of people who will develop cancer this year, and 350,000 people will die of it in this country, very few will have a history of having been exposed to known carcinogenic agents.

What do we know about the OSHA compounds for which there is strong epidemiologic evidence of human carcinogenic activity? Benzidine has been a suspect in "aniline dye cancers" since Heuper's report of 1934; more recently, workers exposed only to benzidine and to no other aromatic amines have been shown to have a higher than normal incidence of bladder cancers (Zavon et al. 1950), mammary tumors (Griswold et al., 1968), leukemia (Zabehzhinskiy, 1970), and other tumors (Pliss, 1965; Pliss et al., 1973).

Dogs develop bladder cancer (Spitz et al., 1950; Bonser et al., 1955); hamsters develop liver-cell and cholangiomatous tumors (Saffiotti et al., 1967); mice develop hepatic tumors (Prokof'yeva, 1971). The epidemiological evidence is very strong linking worker exposure to benzidine and excessive bladder cancer development (Case et al., 1954; Zavon et al., 1973; Goldwater et al., 1965). However, there is no published evidence that liver or other tumors besides those of the bladder, except possibly of the kidney and pancreas (Mancuso and El-Attar, 1967), develop to an excessive degree in benzidine-exposed workers (Table 1).

TABLE 1. ANIMAL AND HUMAN DATA ON BENZIDINE

DOG	HUMAN
200 mg/day, 6 days/week, 15 months then 300 mg/day, 6 days/week, for 45 months. (Total 325 grams)	The dose is unknown in all cases.
3/7 dogs developed bladder carcinomas and papillomas (at 7, 8, and 9 years)	298 cases with known contact with benzidine, β -NA, or α -NA, in British chemical industry, 1921-1950. 17/76 exposed only to benzidine developed bladder cancer. Exposure mean was 18.7 years, range 5-33 years. No increase in other malignancies. 20/639 exposed to β -NA and/or benzidine developed cancer of kidney and bladder; 6 developed pancreatic cancer. 13/25 exposed primarily to benzidine developed bladder tumors, 11 of which were malignant. Exposure period averaged more than 13 years.

β -Naphthylamine, along with benzidine, was suspected as a causative agent in bladder tumors in the aniline dye industry (Heuper, 1934). Workers exposed to β -naphthylamine are said to have a risk of bladder cancer 61 times that of the general, unexposed public (Case et al., 1954).

Dogs were first shown in 1938 by Heuper to develop bladder cancer following subcutaneous and oral dosing with β -naphthylamine. Although metastases were thought not to occur, the development of lung and renal metastases in dogs has been reported. These metastases developed 2-5 years after a 2-year dosing regimen (Harrison et al., 1969). Monkeys

(Conzelman et al., 1969) and hamsters (Saffiotti et al., 1967) have also been shown to develop bladder cancers; other tumors seen include liver and pulmonary cancers, sarcomas, and lymphomas. In man, however, epidemiological evidence exists only for bladder tumors (Table 2).

TABLE 2. ANIMAL AND HUMAN DATA ON β -NAPHTHYLAMINE

SPECIES	RESULT	HUMAN
DOG	<p>4.5 mg/day subcut. for 3.5 months; 8-10 mg/day, for 3 months. Oral administration was added at various doses and times; 13/16 developed bladder lesions, 9 of which were malignant and 4 were premalignant. Tumors developed in 18-24 months.</p> <p>Orally for total dose of 310 grams (200 mg followed by 600 mg/day); 2/4 developed bladder tumors, one of which was malignant.</p> <p>Orally, 400 mg/day for 2 years. 4/4 developed vesical tumors 9-18 months after beginning treatment. Metastases were found in two, in lung and kidney.</p>	<p>262/4622 men employed 6 months or more developed bladder cancer. 55/262 had been exposed only to β-NA.</p> <p>109/376 workers in a dyestuffs plant had bladder malignancies. 17/54 exposed only to β-NA had the tumors. Latency period ranged from 6-38 years.</p>
HAMSTER	In diet, at 1%. 18/39 developed bladder tumors, mostly transitional cell carcinomas.	
MONKEY	6.25 - 400 mg/kg by stomach tube 6 days/week for 5 years. 9/24 developed transitional cell carcinoma in 33-60 months.	

4-Aminodiphenyl is considered to be many more times as potent in causing bladder cancer in the dog than are β -naphthylamine and benzidine (Deichmann and Radomski, 1969). Dogs (Walpole et al., 1954) develop bladder tumors when dosed orally or subcutaneously with 4-ADP. Mice also develop hepatomas (Clayson et al., 1967; Gorrod et al., 1968). Epidemiologic evidence is very strong linking 4-ADP production with an increased risk of bladder cancer (Melick et al., 1955); in fact, 4-ADP may well be more hazardous than the other aromatic amines for man as well as for the dog, although benzidine is said to be much more carcinogenic in man than in the dog (Deichmann and Radomski, 1969). Production of 4-ADP ceased in 1955; however, because bladder tumors often show a latency period of 20 years or longer, cases of bladder cancer in exposed workers may still develop (Melick et al., 1971).

4-Nitrobiphenyl is the parent compound of 4-ADP. When production of the latter ceased, so did that of 4-NBP. The epidemiologic evidence of bladder cancer from this compound is clouded by concurrent exposure to 4-ADP. Dogs

fed with 4-NBP at a dose of 0.3 g three times a week for up to 33 months developed bladder tumors (Deichmann et al., 1958a); at lower doses these tumors did not occur (Deichmann et al., 1965a and 1965b). Experimental work in rats suggests that 4-NBP must be reduced to 4-ADP in order to be active as a carcinogen (Laham, 1960). It is clear, however, that exposure to 4-NBP may lead to bladder cancer in man.

It is interesting to note that we have covered four of the five OSHA compounds mentioned earlier as being strongly implicated in human cancer, and they are all bladder carcinogens in both man and animals, although they possess more varied activity in animals.

Bis(chloromethyl) ether is an extremely potent lung carcinogen in laboratory animals - at doses as low as 0.1 ppm (Laskin et al., 1971).

Recent reports of excessive oat cell carcinoma of the lung have singled out BCME as the causative agent (Lassiter, 1973; Thiess et al., 1973; Sakabe, 1973), possibly present only as a contaminant of chloromethyl methyl ether (Figueroa et al., 1973). The epidemiologic studies with these two compounds (BCME and CMME) are not as complete as would be desired, but the evidence is nonetheless striking (Table 3).

TABLE 3. ANIMAL AND HUMAN DATA ON BIS(CHLOROMETHYL) ETHER

SPECIES	RESULT	HUMAN
MICE	Inhalation of 1 ppm 6 hrs/day, 5 days/week for 82 exposure days. 50 animals were exposed; 47 were autopsied; 37 had lung abnormalities; 26 had lung tumors. Mean number of tumors: 2.89. Control animals had a tumor incidence of 20/49; mean number of tumors per animal was 0.87.	4 cases of lung cancer developed in workers exposed 5 or more years to BCME, mostly undifferentiated oat cell carcinoma (only 0.54 cases would have been expected.)
RATS	0.1 ppm for 6 hours/day, 5 days/week for 101 exposures. Of 19 animals autopsied, 5 had squamous cell carcinoma of the lung, 5 had esthesioneuroepitheliomas, 1 had both tumors.	6/18 people in a research center working with BCME died of lung cancer; 2/50 in the production facility also died within the same period. 5/8 were oat cell carcinomas.
MICE	Newborn Swiss mice injected with 12.5 µl/kg 1 time. 45/100 developed lung adenomas.	
MICE	Skin painting with 2.0 mg in 0.1 ml benzene, 3 X per week for 325 days; 12/20 developed squamous cell carcinomas.	

α -Naphthylamine (Case et al., 1954) and chloromethyl methyl ether (Figuerola et al., 1973) are implicated as human carcinogens, but β -naphthylamine and bis(chloromethyl) ether are probably contaminants of them. It is not possible to state categorically the carcinogenic potential of the pure compounds, although this may be of little practical importance unless the carcinogenic impurities can truly be kept to a very low level.

We have no human data for the other seven carcinogens from OSHA's list and they are presumed to have potential for causing human cancer because of their strong activity in animals. It is not known whether the lack of evidence in man is due to the fact that.

- a) the compound does not cause cancer in man: species variability;
- b) the necessary time between exposure and development of human tumors has not elapsed - it is possible that a latency period in man may be greater than the average life-span;
- c) the proper epidemiological investigations have not been done yet;
- d) the data available are being misinterpreted.

What about some other "known" human carcinogens? We can very briefly discuss asbestos, chromates, nickel compounds, and vinyl chloride.

Autopsy reports first suggested a relationship between asbestosis and lung cancer in 1935. Epidemiological studies published by Doll in 1955 firmly established the link between asbestos and lung cancer, although it had been suspected earlier (Isselbacher et al., 1953). More recent work is being done on the association between asbestos and cigarette smoking (Selikoff et al., 1968).

Asbestos exposure has also been linked to the development of peritoneal and pleural mesotheliomas (Wagner et al., 1960; Enticknap and Smither, 1964; Newhouse and Thompson, 1965; Borow et al., 1967; Borow et al., 1973), primarily following exposure to crocidolite and chrysotile forms of asbestos. The latency period is long - 20-60 years - and the exposure period may have been short - 10 months, maybe less. Exposure need not have been severe.

Rats, when exposed to all forms of asbestos (Wagner et al., 1974) (amosite, anthophyllite, crocidolite, chrysotile), develop asbestosis when exposed 8 weeks or more. Lung tumors were also seen following exposure to various types of asbestos, more frequently after chrysotile, however. Mesotheliomas developed after as short an exposure as one day. Mice have developed pulmonary adenomas of questionable malignancy following chrysotile asbestos exposure (Lynch et al., 1957), but rats seem to be more predictive for asbestos cancers in man.

Workers in various chromate-producing industries have been shown to have an excessive risk of developing lung cancer (Bidstrup and Case, 1956; Baetjer, 1950a and 1950b). The first cases of lung cancer associated with chromate exposure were reported in 1932 in Germany and in 1948 in the U.S. Exposure periods have varied from 4 to 41 years. The specific etiologic agent(s) is not known. Squamous cell carcinoma of the bronchus is most commonly found.

Baetjer attempted to elicit bronchogenic carcinoma in mice and rats by inhalation of chromium materials; however, the results were negative (Baetjer et al., 1959). The experiment was designed to duplicate as much as possible the conditions of a "typical" chromate-producing plant. The mice were exposed four hours per day, five days per week to 1-2 mg/m³ for 24-62 weeks, the rats to 2-3 mg/m³ for up to 101 weeks. There were no significant differences in tumor incidence between experimental and control animals, although the exposed rats did show more lymphosarcomas than did the controls. Further attempts to produce bronchogenic carcinomas in rabbits and guinea pigs, and again in rats and mice, in a similar experiment also failed. There also was no significant increase in pulmonary adenomas nor in lymphosarcomas in rats (Steffee and Baetjer, 1965).

Hueper and Payne (1959) implanted various chromate compounds intramuscularly and intrapleurally in rats; sheet fat pellets were used as a vehicle. Calcium chromate, sintered calcium chromate, and sintered chromium trioxide induced sarcomas at the implant site in 57-79% of the animals. Similar results were obtained with mice, but when tricaprylin was used as a vehicle, only one tumor developed (Payne, 1960).

The relation of the foregoing animal data to the development of squamous cell carcinoma of the bronchus in man is not clear. Finally, about five years ago, Laskin (1970) using implantation of cholesterol pellets containing calcium chromate in the bronchus of rats has produced squamous cell carcinoma of the bronchus. Dose-response information is not available, however. The literature on chromium compounds was reviewed recently by a Committee of the National Research Council (1974).

Cancer of the nasal cavities and paranasal sinuses had been associated with nickel refinery work in 1932 in England; by 1937 lung cancer was also being found in excess among refinery workers in Germany. By 1949 it was noted that most of the cancer victims in England had begun their employment before 1924 (Morgan, 1958). In 1958, Williams reported the pulmonary pathology of five nickel refinery workers, four of whom had pulmonary carcinomas, three of which were squamous cell carcinomas and the fourth, alveolar cell carcinoma. Of these four, three had started work after 1924. A later, more extensive study was carried out on 845 men employed prior to 1944 in a nickel refinery. This study showed a mortality from cancer of the nasal cavities greater than 364 times the national average in those starting employment prior to 1925; no deaths from this cancer occurred in men who started after 1925. In late 1924, changes occurred in the refinery process: less dust, some personal protection, and arsenic-free sulfuric acid was introduced. What the critical change was, we don't know. Mortality from pulmonary cancer in those starting prior to 1925 was 7.5 times the national average, while in those employed after 1925, it was 1.3 times the average (Doll et al., 1970). Similar excesses have been found in other countries. The responsible agents are not known.

Experimental induction of pulmonary carcinomas has succeeded in rats with nickel dust (Hueper, 1958), and nickel carbonyl (Sunderman et al., 1959). Various authors have reported carcinomas following intramuscular and intravenous injections of nickel compounds (Sunderman and Donnelly, 1965; Maenza et al., 1971). Rhabdomyosarcomas are seen in a high percentage of rats following intramuscular or intravenous injection of metallic nickel or nickel subsulfide (Friedmann and Bird, 1969; Heath and Daniel, 1964). An in-depth review of the literature on nickel and nickel compounds is currently under way by a Committee of the National Research Council.

In a report in 1970 by Viola et al., Wistar rats were exposed to vinyl chloride vapors at 30,000 ppm for 4 hours/day, 5 days/week for 12 months. The first sign of ill health other than lassitude, was the appearance in some of the animals of a para-auricular mass which eventually ulcerated, and tumorous tissue formed on the surfaces. Almost all of the animals developed tumors of the skin; 5/16 developed bone tumors (osteochondroma). Lung tumors developed in 6/16 and were mainly adenocarcinomas; there was one epidermoid tumor.

Maltoni at the Second International Symposium on Cancer Detection and Prevention, Bologna, April 9-12, 1973 recommended that

"Epidemiological evaluations and medical controls should be undertaken on exposed workers. Would such neoplastic neoplasia unhappily be found in man, given the rarity of these tumours in both animals and humans, it would be a precise indication of the high value of experimental testing in predicting the oncogenic potential of environmental agents and would strengthen the recommendation on the necessity of such bioassays before any new industrial compound is produced and widespread in large scale."

In 1974 Maltoni reported on Sprague-Dawley rats exposed to 10,000, 6,000, 2400, 500, and 250 ppm vinyl chloride; the animals developed nephroblastomas, angiosarcomas of the liver and other sites, ovarian tumors, and zymbal gland adenomas; at all but the lowest dose, zymbal gland carcinomas with metastases to the lung also developed. These tumors generally do not occur spontaneously in these rats.

At a meeting in Rome on March 9, 1974, a paper by Maltoni and Lefemine was presented by S. G. Favilli; it stated that one and one-half years before, when it became clear that vinyl chloride was carcinogenic, "...we gave immediate notice of our findings not only to the Society of the European Cooperative Group but also to the other major societies of producers in the world" (Maltoni and Lefemine, 1974). It is unfortunate that the regulatory agencies in this country did not know of the liver tumors in experimental animals until after they were informed of the liver tumors in humans working with vinyl chloride (Key, 1974). The data that Maltoni and Lefemine developed is shown in Table 4.

It is seen that tumors have developed in mice and hamsters as well as rats. Very recent results (mid-June) from Maltoni's laboratory are showing tumors at 50 ppm. Sixty-four rats were exposed to 50 ppm vinyl chloride for one year; they showed extensive, varied tumors. The three animals which survived one-year exposure had liver and intra-abdominal angiosarcomas and nephroblastoma. Mice exposed to 50 ppm for seven months showed liver, pulmonary, mammary and skin tumors (OSHA, 1974).

The first case of angiosarcoma of the liver linked to occupational exposure to vinyl chloride was identified in January 1974. The patient had died in 1973 after 28 years of exposure; the autopsy diagnosis was angiosarcoma of the liver. Retrospective study revealed three other cases, with deaths in 1964, 1968, and 1973. Exposure had lasted 15-20 years. A sixth case was diagnosed at biopsy in February; his exposure had been for 12 years (Block, 1974).

TABLE 4. EXPOSURE OF RATS AND MICE TO VINYL CHLORIDE*

TABLE 4. EXPOSURE OF RATS AND MICE TO VINYL CHLORIDE*

Experiment Number	Route	Dose	Duration	Species	Total No.	Results
BT1	Inhal.	10,000, 6,000 2500, 250, 50 ppm	4 hrs/day 5 days/week for 52 weeks	Sprague- Dawley rats	577	<p>At 10,000 ppm, 69 were exposed; none survived at 131 weeks. There were 27 tumors total; 6 were liver angiosarcomas, 13 zymbal gland carcinomas.</p> <p>At 6000 ppm, 72 were exposed; none survived. There were 21 tumors total; 11 were angiosarcoma of the liver; 5 zymbal gland carcinomas.</p> <p>At 2500 ppm, 74 were exposed; none survived. There were 21 tumors total; 9 liver angiosarcomas, 2 zymbal gland carcinomas, 6 nephroblastomas.</p> <p>At 500 ppm, 67 were exposed; 0 survived. There were 15 tumors total; 7 liver angiosarcomas.</p> <p>At 250 ppm, 67 were exposed; 1 survived. There were 9 tumors total, 5 nephroblastomas, 2 angiosarcomas of the liver.</p> <p>At 50 ppm, 64 were exposed; 3 survived; there were no tumors.</p> <p>At this level, there were 96 tumors; 35 were angiosarcomas of the liver.</p>
BT3	Inhal.	10,000, 6,000, 2500, 500, 250, 50 ppm	4 hrs/day, 5 days/week for 17 weeks	Sprague- Dawley rats	550	<p>At 10,000 ppm, 60 were exposed with 36 survivors at 60 weeks. There were 3 zymbal gland tumors.</p> <p>At 6000 ppm, there were 48 survivors of 60. There was one zymbal gland tumor.</p> <p>At 2500 ppm, there were 54 survivors of 60. There were no tumors.</p> <p>At 500, 250, and 50 ppm there were no tumors; almost all of the animals survived at 60 weeks.</p>
BT4	Inhal.	10,000, 6,000 2500, 500, 250, 50 ppm	4 hrs/day 5 days/week for 30 weeks	Swiss Mice	510	<p>At 10,000 ppm, there were 60 exposed with 24 survivors at 35 weeks. There were 12 pulmonary tumors, 4 mammary carcinomas, 3 tumors of other sites.</p> <p>At 6000 ppm, there were 60 exposed with 29 survivors at 35 weeks. There were 12 pulmonary tumors, 3 mammary carcinomas, 1 liver angiosarcoma, 2 tumors of other sites.</p> <p>At 2500 ppm, there were 33 survivors of 60. There were 7 pulmonary tumors and 2 mammary carcinomas.</p> <p>At 500 ppm, there were 38 survivors. There were 7 pulmonary tumors and 2 liver angiosarcomas.</p> <p>At 250 ppm there were 41 survivors. There were 4 pulmonary tumors and 1 mammary carcinoma.</p> <p>There were no tumors at 50 ppm.</p>
BT5	Trans-placenta	10,000, 6,000 ppm	4 hrs/day for 7 days (on days 12-18 of gestation)	Sprague- Dawley rats	146	<p>At 10,000 ppm, mothers had no tumors. Newborn had 1 subcutaneous angiosarcoma.</p> <p>At 6000 ppm, mothers had no tumors. Newborn had 1 subcutaneous angiosarcoma.</p>
BT6	Inhal.	30,000 ppm	4 hrs/day, 5 days/week	Sprague- Dawley rats	60	There were 2 zymbal gland carcinomas.

*From Maltoni and Lefemine, 1974a and 1974b.

A 57% excess mortality from cancer has been seen in vinyl chloride polymerization plants (Key, 1974).

Twenty-one cases of angiosarcoma of the liver in vinyl chloride polymerization workers have been identified world-wide. Additional cases have been found; one worked in vinyl chloride monomer plant; another filled spray cans with vinyl chloride propellant; two more cases worked in vinyl-cloth plants; two other cases have been identified as living near facilities using vinyl chloride or polyvinyl chloride (Key, 1974; Wagoner, 1974). It is interesting to speculate on whether or not these tumors would have been discovered if they had not been so rare and unusual.

It is not possible to state the dose responsible for these cancers in man; and since no "no response" level has been identified in animals, extrapolation of a "safe level" from animal to man cannot be an easy decision. We are fairly certain it will not be zero and quite certain it will not be 50 ppm.

DISCUSSION AND CONCLUSIONS

From these observations on some of the best known and most thoroughly studied compounds we can draw some conclusions about the value of animal studies in predicting human cancer. We find that animals can indeed be highly predictive of carcinogenicity in man. In some cases, as with vinyl chloride, they are predictive as to the site of tumor development and the kind of tumor. In other cases, they are not predictive of the site or kind of tumor, but only suggest tumorigenesis. Unfortunately, the animal data are not always of sufficient quality and, as with any living system, we are always going to have some "false positives" and some "false negatives."

It does not appear that animals are predictive of the exact dose producing cancer in man. This may be due in part to our lack of knowledge of the actual effective human dose. It does seem that a relative ranking based on the minimum dose producing cancer in animals may be predictive of those compounds most likely to be carcinogenic in man. This, of course, implies that dose-response studies have been done.

It is also clear that the reliability of the prediction from animals to humans is enhanced by production of tumors in more than one animal species, preferably in the rat and two other species.

Considering the variability among biological systems it would not be surprising that the animals occasionally failed to predict known human carcinogens. Yet we are only aware of one "false negative," arsenic, as we have already discussed. Even in this case there is one report of increased leukemia and malignant lymphomas in pregnant Swiss mice injected with arsenic (Osswald and Goerttler, 1971). The other side of the coin is a somewhat different story. There are roughly 1000 compounds producing cancer in animals for which we have no evidence of their carcinogenicity in man. These "false positives" constitute materials which require great care in their use and continual surveillance of those at risk. The greater the volume of animal data, especially dose-response information, the more certain we can be about exposing humans to a set level.

Finally, we would like to raise one more point. Why have we so often discovered the carcinogenicity of a compound first in humans and then later in animals as is the case with asbestos, vinyl chloride, benzidine, β -naphthylamine? Do we lack faith in our toxicology data? We trust our data for other responses such as CNS effects, respiratory irritation, liver injury, kidney damage, and blood dyscrasias. Why not for carcinogenicity? Is it because the epidemiologists do not have enough information and precise enough methodology to confirm our "false positives?" Of the 350,000 deaths from cancer expected this year, very few will have an identifiable causative agent. Is smoking that much of a complicating factor? Is man that much more sensitive that one or two exposures, so slight as to go unnoticed and unremembered, are causing these human tumors? Are we nictitating because of economic pressures?

In looking at the various kinds of cancer, especially those from occupational exposures, there are three factors pertinent to our discussion. First, there is a long induction period, sometimes as much as 20 or 30 years, between the first exposure and the first indication of cancer. This means that whenever a chemical is introduced in large quantities into the workplace or the public arena we risk initiating an accumulating backlog of cancer cases that will come crashing down on our heads many years later as with asbestos and vinyl chloride and β -naphthylamine.

It is extremely doubtful whether we can ever be 100% sure that even the best animal data are not false positives or false negatives unless they are put to the final test of extensive human exposure. Therefore, it behooves us to minimize the risk involved by adequate animal carcinogenic testing prior to human exposures. The greater the anticipated human exposure in terms of dose, duration, and number at risk, the greater must be the extent of our animal data.

Second, in all of these instances of known chemical carcinogenesis in humans that we have reviewed, the animal data have been generated after the human data. We must reverse this process and get predictive animal data before humans are exposed. Furthermore, we should be initiating studies on those chemicals currently in high volume use for which we do not have predictive animal data.

With this tremendous task ahead of us we have an equally pressing need to develop more efficient test procedures of equal or greater reliability. Some promise rests in tissue cultures and in the adaptation of some of the mutagenic procedures. The correlation between mutagenesis and carcinogenesis is continuing to improve.

Third, what do we do with the data we now have and the data we will be accumulating on carcinogenesis in animals by chemicals for which we have no corresponding information in humans? It is obvious from our foregoing study that data in several species are more likely to be predictive of human cancer than if only one highly susceptible species or strain is used. We badly need criteria for evaluating or ranking the predictive value of animal data for human cancer.

In conclusion, animal data can be predictive of carcinogenicity for humans. What are we waiting for? Let's get on with the job and have some faith in the results of our own experimental data.

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SUITABILITY OF ANIMAL SPECIES AND STRAINS
FOR TESTING CARCINOGENIC RISK FOR MAN

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In recent years the health sciences have recognized the unique contributions attributable to studies in comparative medicine. However, the many diseases that occur spontaneously in animals with similar counterparts in man have been only superficially studied or have not been recognized. Further, induced disease has gained wide acceptance as a means for investigating pathogenesis and mechanisms impossible to study in man; carcinogenesis studies are a good example of the value of the experimental animal approach.

Testing for the safety of chemicals to which human population groups are exposed requires animals, and important considerations in such studies include the choice of animal model(s), the diet, housing and handling and the interpretation of results. The safety of people testing chemicals requires conditions with the least likelihood for human exposure; an important factor in this regard is route of exposure and method of administration of the test substance. Chemicals may be administered in the diet, by gastric intubation, by intraperitoneal injection, intratracheal intubation, by aerosol or by other means. The most practical route of exposure, in keeping with safety, is that which is closest to the manner in which humans may be exposed.

Selections of suitable models are made from the large number of species and strains available and for one reason or another are chosen on a basis of economy, ease of handling of significant numbers, target organ(s) of interest, known biologic response to other classes of chemicals, potential for extrapolating results to man, and for many other reasons. It is generally accepted that at least one rodent and one non-rodent species should be used in most carcinogenesis studies.

We have explored the use of a number of species of laboratory animals in carcinogenesis studies and selected examples are discussed in this paper. Problems associated with the use of mice in safety evaluations are alluded to and the use of animal studies to identify potential human carcinogens is pointed out.

MODEL FOR HUMAN LUNG CANCER

Lung tumors have been induced in small laboratory animals by transfixing lungs with carcinogen-impregnated threads, with (Andervont, 1937) or without (Kuschner et al., 1956) concurrent intrabronchial implantation of carcinogen-impregnated wire pellets, by intravenous injection of carcinogens and cigarette smoke condensates into the lung (Blacklock, 1957), and intrabronchial implantation of 106 Ruthenium pellets (Laskin et al., 1963). These methods produce considerable tissue injury, making it difficult to differentiate specific (carcinogenic) from nonspecific (necrosis, inflammation) tissue responses. Intrabronchial instillation of carcinogen suspensions induces lung tumors in rats, but histologic analysis is complicated because of the high incidence of chronic pneumonia in this species (Saffiotti et al., 1964). Lung tumors are produced in dogs trained to smoke cigarettes, but this model is limited because of the time and expense involved per animal (Hammond et al., 1970; Auerback et al., 1970).

We have modified a model first developed by Saffiotti et al. (1964), wherein hamsters were used because of their low incidence of spontaneous lung tumors and relative freedom from chronic respiratory infections. The carcinogenic treatment consists of a series of weekly intratracheal doses of 3 mg of benzo(a)pyrene (BP) adhered to 3 mg hematite (Fe_2O_3) in 0.2 ml of 0.15 M sodium chloride. The hamsters, initially weighing 100 g, are anesthetized with halothane in a semi-closed circuit, multichambered, induction box (Figures 1 and 2) designed in our laboratory (Smith et al., 1973). Large numbers of animals can be anesthetized without difficulty and with very low mortality from anesthetic accidents using this system. Prior to instillation, anesthetized animals are suspended vertically and held against a plastic stand by an elastic band (Figure 3). The carcinogen suspension is deposited intratracheally through a 19 gauge, 7 cm, blunt-tipped needle, bent 150 degrees 3.5 cm, from the tip. Animals treated in this manner develop tumors when fed either commercial natural product diet or a semi-synthetic diet (Table 1) but the use of the latter makes it possible to evaluate the nutritional status and its effect on the disease process in a more precise manner. The carcinogen-hematite particles are uniformly distributed throughout the lungs after instillation. They penetrate the respiratory duct and alveolar walls where they are phagocytized by macrophages and thus gain entrance into the respiratory tissue with neither extensive tissue damage nor destruction of the normal mucociliary barrier. Squamous metaplasia, hyperplasia and cellular atypia occur focally in the respiratory epithelia six weeks after the first BP instillation. Papillomas and polyps develop as early as nine weeks, and malignant tumors begin to appear 10 weeks after the final instillation. About 30% of treated hamsters live more than one year after the BP instillations and most deaths after the completion of the instillations are due to respiratory tumors. The overall tumor incidence is approximately 80% of those receiving 12 BP instillations.

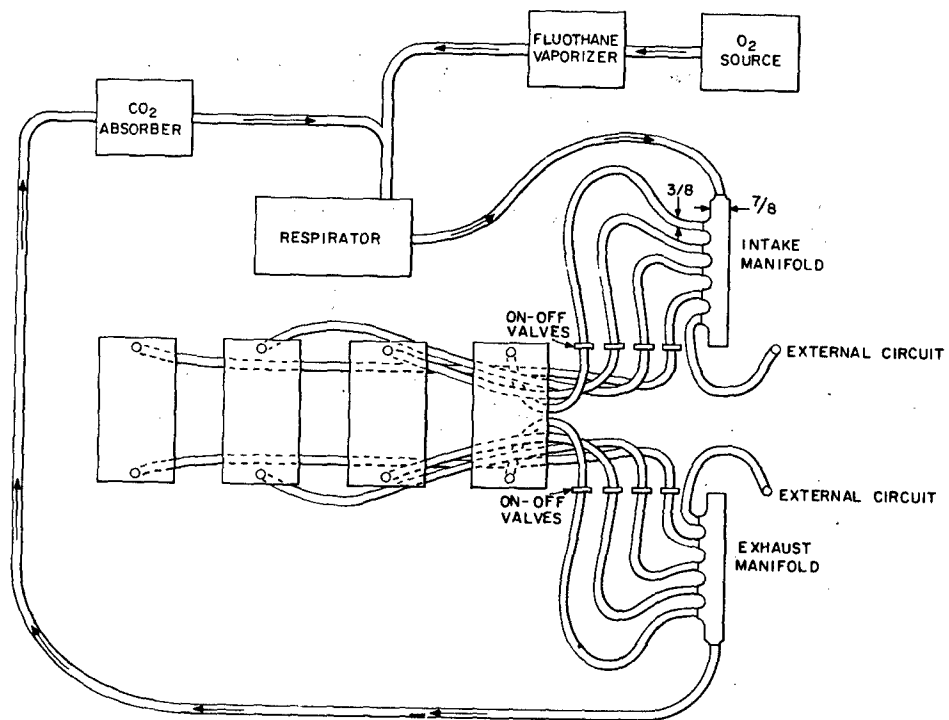


Figure 1. Diagram of gasing circuit.

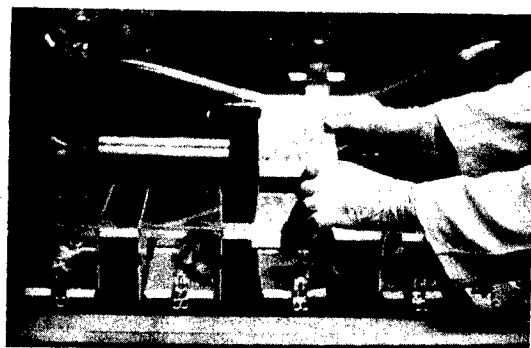


Figure 2. Anesthesia chamber in operation.

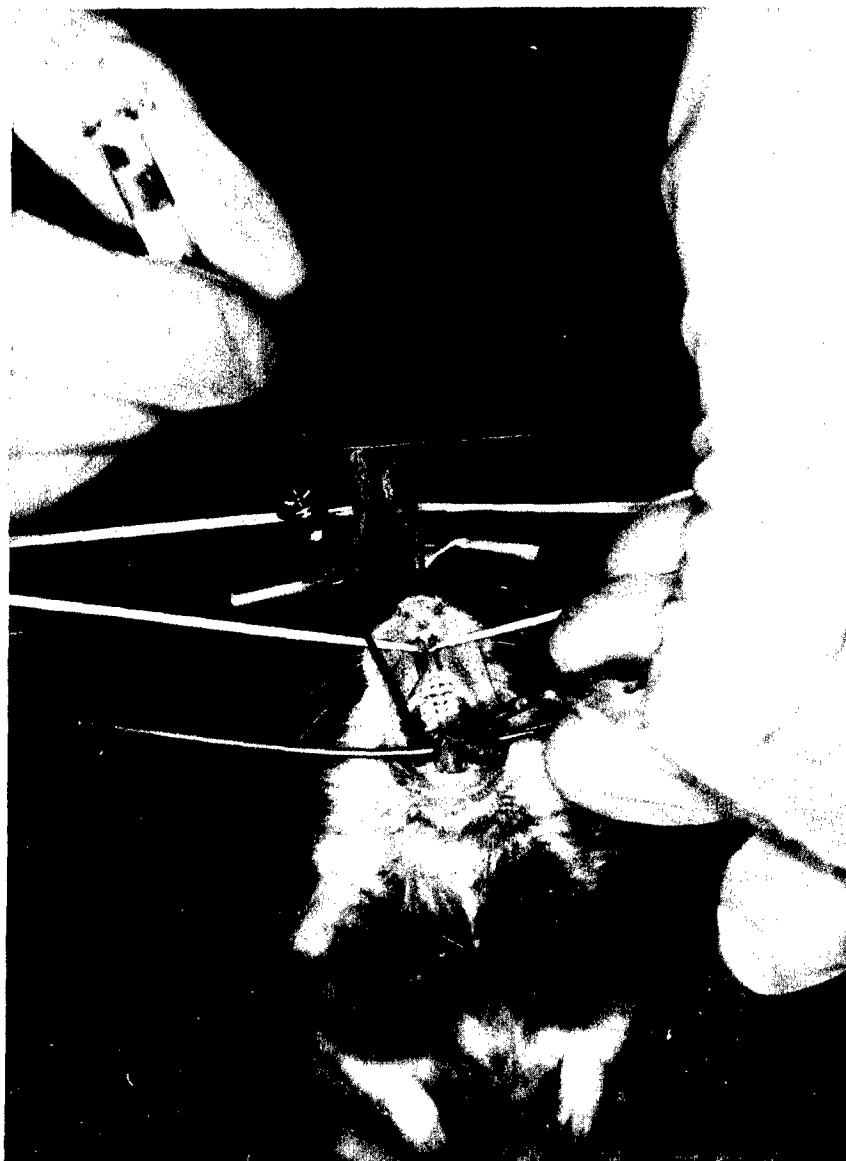


Figure 3. Depositing carcinogen into trachea of anesthetized hamster.

TABLE 1. COMPOSITION OF SEMISYNTHETIC DIET*

	<u>per kg</u>
Vitamin Free Casein	230 g
Cornstarch	400 g
Sucrose	217 g
Rogers-Harper's Salt Mix**	50 g
Cellulose	50 g
Vitamin Mix***	20 g
Choline Chloride	3 g
Vitamin B ₁₂	50 μ g

* Suspended in equal volume 3% agar solution (15)

** Purchased from Nutritional Biochemical Corporation, Cleveland, Ohio 44128.

*** 1 kg of vitamin mix contains: 0.50 g menadione, 0.20 g riboflavin, 0.40 g pyridoxine HCl, 1.0 g calcium pantothenate, 0.5 g folic acid, 250 g nicotinic acid amide, 12.50 g inositol, 0.30 g vitamin D₂, 22.50 g alpha-tocopherol acetate (500 IU/g), 0.2 g vitamin A acetate, and 959.2 g sucrose.

Hamsters treated in this manner develop lung tumors of the same cell types seen in man. Bronchogenic carcinoma is the most common of the induced malignant tumors and it is morphologically indistinct from the human tumor (Figure 4).

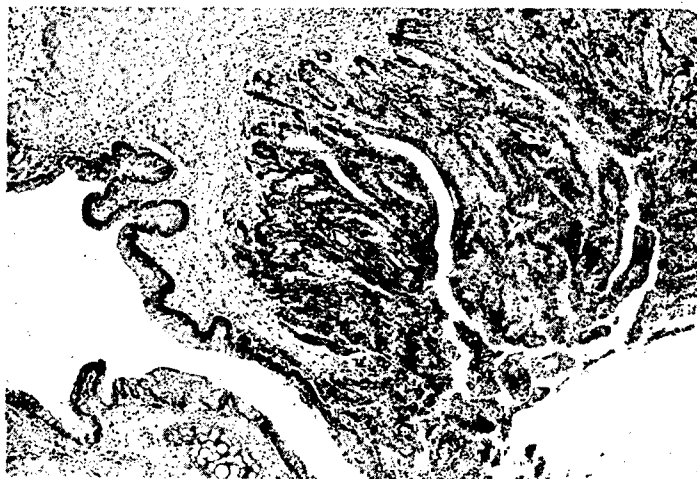


Figure 4. Bronchogenic carcinoma at bifurcation of trachea.

Squamous cell carcinomas, undifferentiated carcinomas and mixed tumors of the larynx and trachea, morphologically similar to those seen in man, are also found in high incidence (Figures 5 and 6).

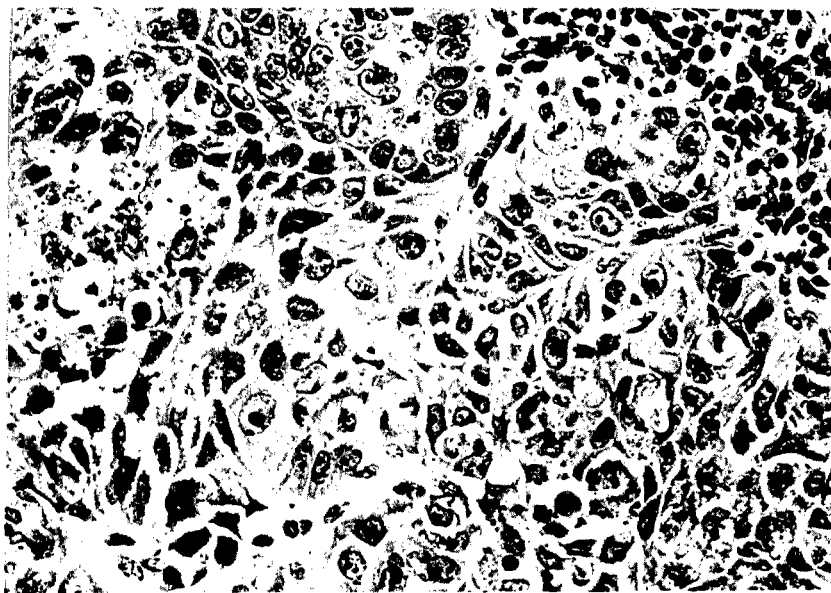


Figure 5. Squamous cell carcinoma of hamster lung.

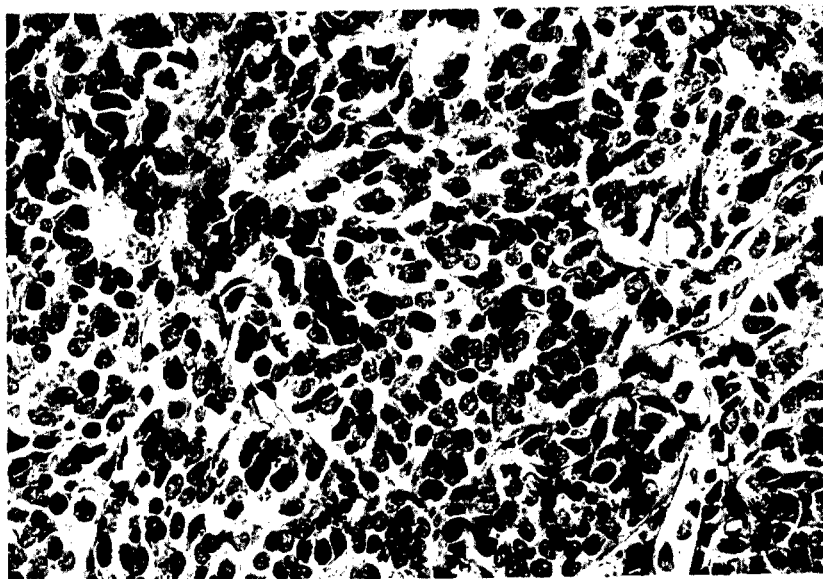


Figure 6. Undifferentiated carcinoma lung of hamster.

Cytologic examination of sputum can be used to detect and monitor the progression of the disease in hamsters and man (Schreiber and Nettesheim, 1972; Koss, 1968). Induced respiratory tumors in hamsters do not metastasize as readily as the human counterpart apparently because the tumors kill the animals before this can occur. Table 2 indicates typical results obtained using this model and illustrates the utility of the hamster for safety testing of environmental chemicals.

TABLE 2. RESPIRATORY TRACT TUMORS IN HAMSTERS
INDUCED BY BENZO(A)PYRENE

<u>Group Number</u>	<u>Number Hamsters</u>	<u>% with Tumors</u>	<u>Average Number Tumors/ Tumor-bearing animal</u>
1	57	59	1.85
2	58	78	1.60
3	58	74	1.72

Another animal model used extensively for safety evaluation of potential carcinogens as well as for investigations into pathogenesis and mechanisms of liver cancer is the aflatoxin treated rat.

MODEL FOR LIVER DISEASE AND CANCER

Although primary hepatic carcinoma is relatively rare in the United States, the overall rate of about 2.4 deaths/100,000 (Higginson, 1969) is sufficient cause for concern for clinicians and for research scientists engaged in etiologic and pathogenetic investigations of cancer induction. Liver cell cancer is a major problem in some population groups in Africa, South China, Hawaii, Rumania and elsewhere (Doll, 1967; Cook and Burkitt, 1971) and the increasing incidence of hepatitis and alcoholic cirrhosis in the United States may be followed by an increase in primary hepatic carcinoma. Utilization of this model, which permits one to investigate the induction of uncomplicated liver cell carcinoma as well as the effect of dietary or other conditions imposed on tumorigenesis, may yield information pertinent to the development of diagnosis and control of the human disease.

The family of aflatoxins were isolated from peanut meal diets which caused hepatic necrosis in domestic animals and poultry in 1961-62 (Newberne, 1965). They were shown to be metabolites of some strains of Aspergillus flavus and the ubiquitous nature of the molds and their metabolites, along with the demonstration in animals that aflatoxins are carcinogenic, have resulted in speculation that these compounds are involved in the etiology of liver disease including hepatocellular carcinoma in man (Wogan, 1968; Newberne et al., 1964; Newberne and Butler, 1969). The availability of chemically pure

aflatoxins and well-defined techniques for inducing liver cell carcinoma in rats provide the model to test this and other hypotheses of hepatic oncogenesis.

Weanling rats of the Charles River CD strain, Fischer strain and Wistar strain fed low dietary concentrations of aflatoxin B₁ (0.1 to 1.0 ppm) develop liver cell carcinoma after 12 to 18 months. An alternate method is to give 15 daily doses intragastrically of 25 micrograms each in 0.1 ml volume of dimethylsulfoxide (DMSO). Clinical signs are generally limited to a slight decrease in food intake during exposure only to intubated aflatoxin B₁; low dietary concentrations elicit no clinical response. With progress of the neoplastic process, serum alkaline phosphatase increases above normal but other hematologic measurements fall within normal range; when the liver tumor is well advanced, signs and symptoms associated with liver failure may be observed. The liver of rats exposed to carcinogenic doses of aflatoxin B₁ reveals a variety of biochemical changes, generally related in nucleic acid and protein synthesis, particularly in the hepatocyte nucleus and nucleolus.

Liver tumors induced by aflatoxin B₁ are multicentric hepatocellular carcinomas without cirrhosis or significant fibrosis (Figure 7).

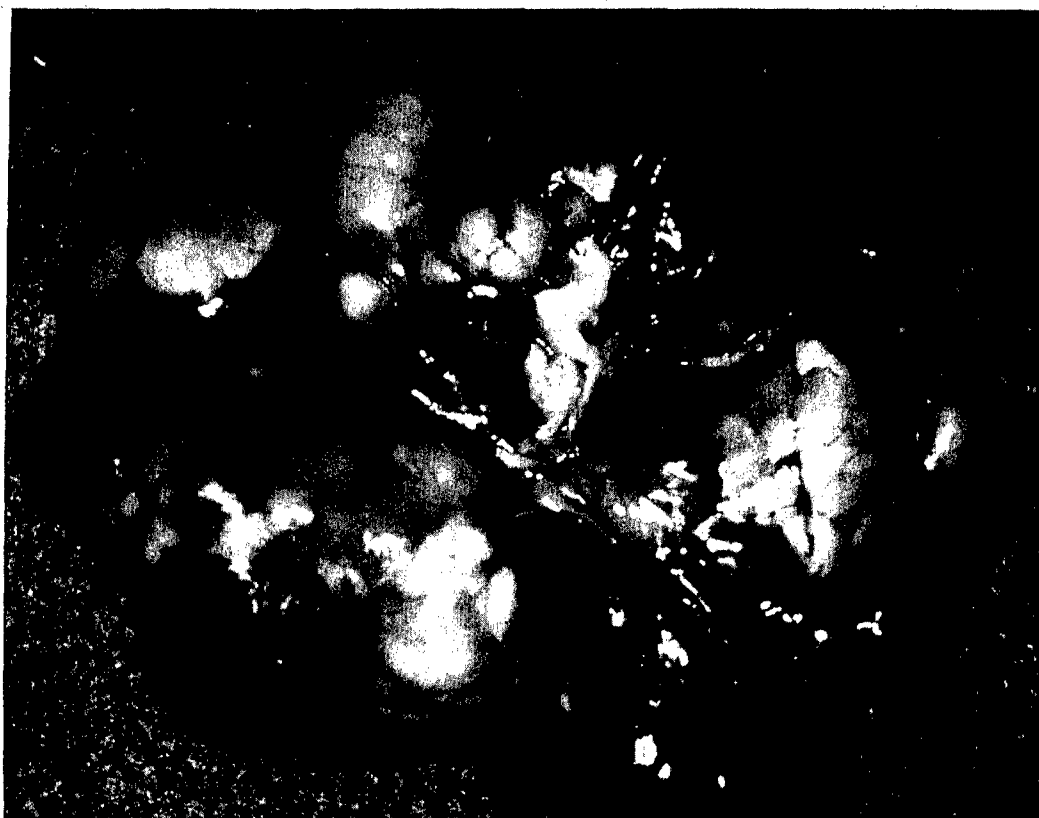


Figure 7. Gross appearance liver cell cancer in rat dosed with aflatoxin.

Tumor nodules are soft, friable and yellowish-gray in color; hemorrhage and necrosis are usually present in more advanced lesions. Microscopically, a trabecular pattern is most often observed (Figure 8), but anaplastic types do occur.

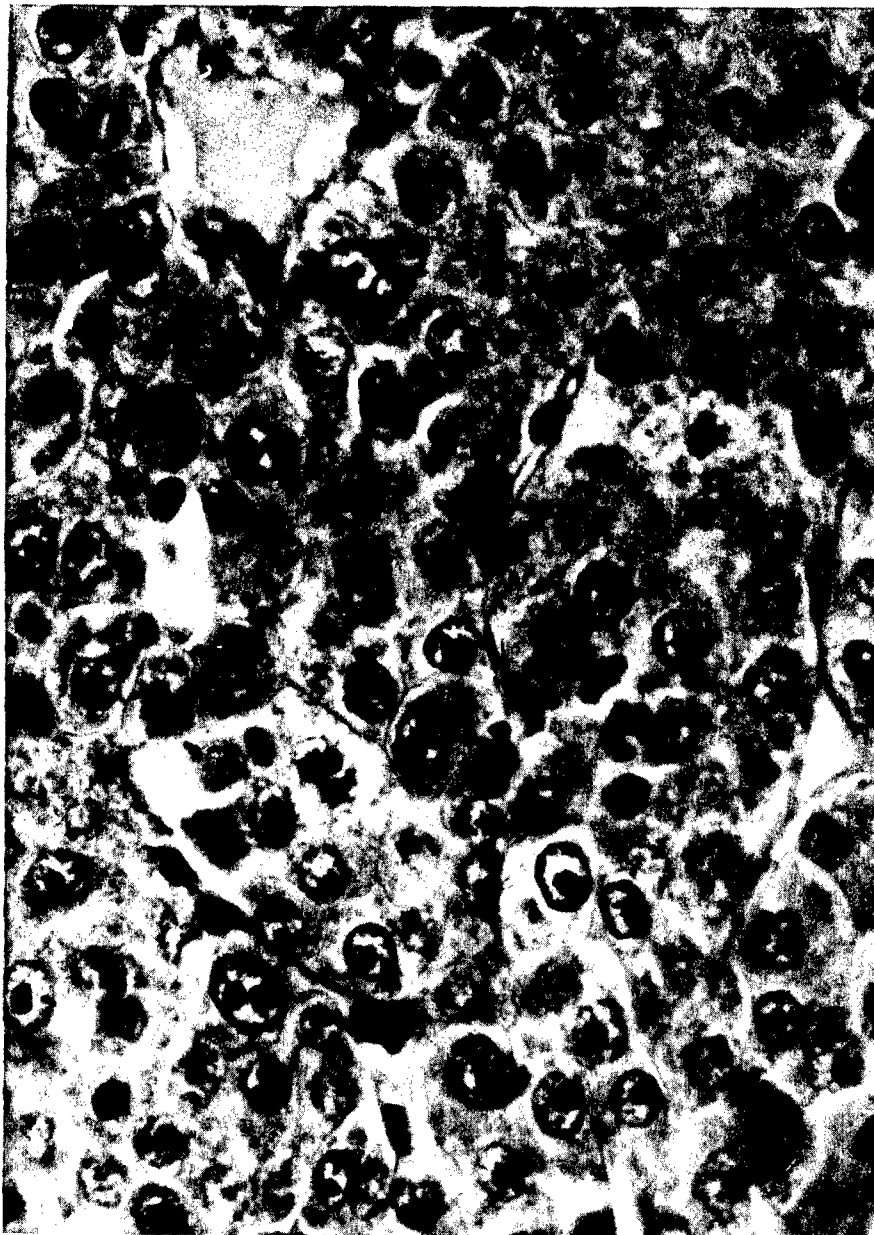


Figure 8. Microscopic appearance of aflatoxin induced liver cell cancer.

Extension of the tumor to the mesentery and peritoneum is often observed and metastasis to the lung is common (Figures 9 and 10).



Figure 9. Gross appearance of metastasis in lungs from animal with liver cancer.

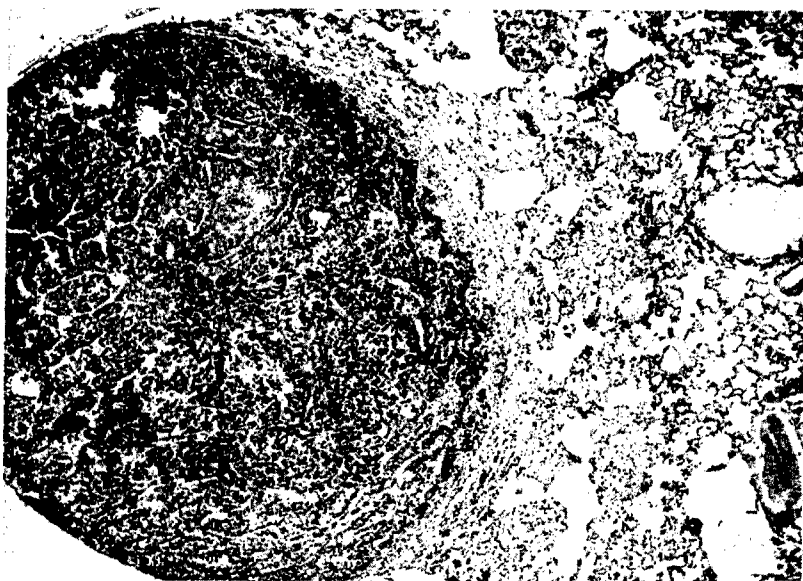


Figure 10. Microscopic appearance of lung metastatic lesion from liver cancer.

Whether the exposure is to a single LD₅₀ of aflatoxin B₁ (about 6 mg/kg body weight) or to smaller, repeated doses by intubation or in the diet, the pattern of changes in the liver appear to be about the same and the sequential changes observed in the liver during tumorigenesis have been described (Newberne and Wogan, 1968). Bile duct hyperplasia, parenchymal cell damage, necrosis and megalocytosis are the most prominent early lesions; these changes usually regress with time. Focal hyperplasia of basophilic parenchymal cells and foci of large, pale eosinophilic cells are seen in the early stages; these are followed by nodule formation and, ultimately, liver cell carcinoma.

The signs and symptoms of aflatoxin B₁-induced liver cell carcinoma in the rat are similar to uncomplicated, primary hepatocarcinoma in man. There is a steady, silent progression of the neoplasm and its extensions until a large nodular mass can be palpated through the abdominal wall. When hepatic function is impaired, jaundice, bleeding or other signs of liver failure, bronchopneumonia and other complications may be seen, but these cases are in the minority in the rat model in contrast to the clinical picture of hepatocarcinoma in man. An occasional case with an episode of hemorrhage and rapid, subsequent death is seen in the rat but these are relatively rare. A notable difference between rat and man and one which probably indicates complex effects of the human environment, is the absence of cirrhosis in the rat.

The several different strains of the laboratory rat offer varying advantages and investigators can choose from a wide selection, part of which is set forth in the Catalogue of Rodents published by the National Institutes of Health (Publication No. 74-606).

MODEL FOR LARGE BOWEL CARCINOGENESIS

About seventy-five thousand Americans annually have a diagnosis of cancer of the large bowel and in some population groups this appears to be on the increase (Burbank, 1971). This form of cancer is the most common internal cancer affecting population groups in the United States and causes about 45,000 deaths each year (McKittrick, 1968), second only to the number of deaths caused by lung cancer. Little is known about the etiology of colon cancer but long-standing ulcerative colitis and some forms of polyps have been implicated (Goldman et al., 1970).

Cancer of the colon probably begins as a local lesion varying from small, ulcerating, firm lesions with elevated margins to the constricting, napkin-ring sclerotic lesions often seen in the left colon; later a large fungating growth protruding into the gut lumen is seen. Part or the whole of the circumference of the bowel is involved and often a large portion of the gut is a rigid, narrow tube. The tumors may be multiple and located in a small area or separated by tracts of normal appearing mucous membrane. Because of a paucity of definitive information about the etiology and pathogenesis of colon carcinoma, an animal model is very useful for studies about this important neoplasm.

Cancer of the large bowel and other gastrointestinal tract neoplasms have been induced in the mouse, rat and hamster by administration of 1,2-dimethylhydrazine (DMH) (Wiebecke et al., 1969; Druckrey et al., 1967; Rogers et al., 1973). The administration by gastric intubation of ten weekly doses each of DMH, dissolved in physiological saline, 30 mg/kg body weight, to weanling rats results in virtually 100% incidence of colon carcinomas within five to seven months.

In the rat, symptoms often go unobserved but close observation may detect visible blood on feces, but better clinical evidence for advancing tumor growth can be obtained by testing feces for occult blood. The general clinical condition of the tumor-bearing rat does not usually deteriorate until the tumor is far advanced and gross manifestations are of little help in estimating stage of tumor induction.

Post-mortem examination reveals tumors, single or sometimes multiple (Figure 11) located usually in the mid to terminal portion of the colon. They may be polypoid, ulcerated, necrotic and bleeding or polyp-like protuberant vegetations or intramural growth. They bleed, obstruct the gut lumen, result in intussusception and, in general, behave much like those observed in man. On section, normal appearing gut wall is often seen in continuity with disorganized neoplastic tissue but polyps form and fronds of neoplastic cells appear to arise from the epithelium (Figure 12) and invasion of the stalk of the polyp is observed. Sharp transition from normal to undifferentiated cells is common (Figure 13) and drastic mutations within a limited group of cells are often seen. Invasion of the gut wall (Figure 14) and regional lymph nodes is observed depending on the stage of neoplastic development.



Figure 11. Gross appearance of colon cancer in rat treated with dimethylhydrazine.



Figure 12. DMH-induced polypoid colon carcinoma with invasion of stalk.



Figure 13. Colon carcinoma with sharp transition between normal and neoplastic epithelium.

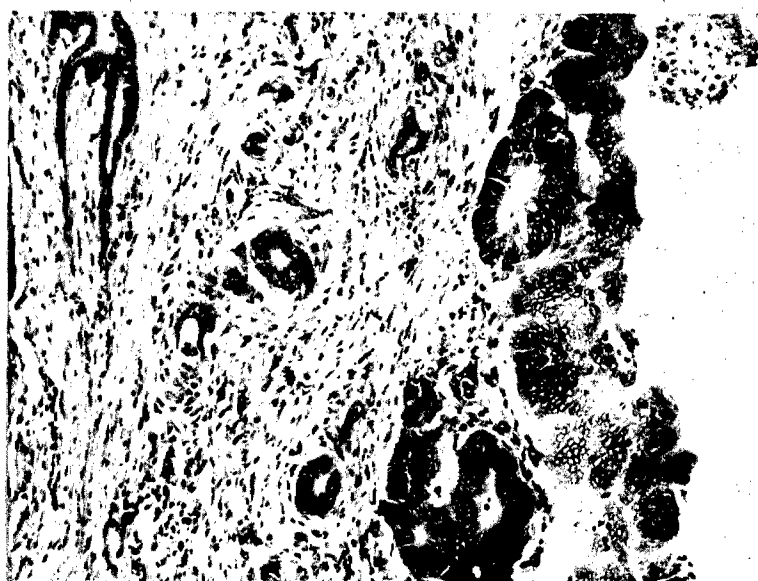


Figure 14. Colon cancer invading gut wall.

These examples of models for carcinogenesis and safety testing have been published or are in press and are only three of more than 50 which have been described and published by the Registry of Comparative Pathology of the Armed Forces Institute of Pathology (Smith et al., in press; Newberne and Rogers, 1973a; Newberne and Rogers, 1973b; Jones et al., 1974). They serve to illustrate the utility of laboratory species in testing programs.

THE USE OF MICE IN SAFETY TESTING

Some species, particularly mice, are less useful for testing and more useful for basic research into pathogenesis and mechanism. The various strains and substrains of mice have been developed for specific purposes and have served well in helping to understand mechanisms of carcinogenesis. In testing programs, however, mice have created more problems than they have solved because of the high incidence of liver lesions which develop in most strains and substrains without treatment and under standard laboratory conditions. Table 3 lists spontaneous hepatic tumors observed in C₃H mice in different laboratories by different investigators. Table 4 refers to spontaneous liver nodules in other strains and substrains.

TABLE 3. SPONTANEOUS MOUSE HEPATIC TUMORS (HEPATOMAS)

<u>Substrain</u>	<u>Numbers</u>		<u>Incidence %</u>		<u>References</u>
	<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>	
<u>Strain C₃H</u>					
--	20	10	20	0	Shimkin (1940)
--	320	--	26	-	Edwards & Dalton (1942)
--	19	--	18	-	Silverstone (1948)
--	102	--	85	-	
f	96	--	71	-	Heston et al. (1960)
e	79	--	78	-	
f	108	--	57	-	
Y/AYA	63	--	100	-	Heston & Vlahakis (1961)
Y/A _a	94	--	88	-	
eB/Fe	134	134	7	7	Davis & Fitzhugh (1962)
AHe	10	--	30 (20m*)	-	Takayama & Oota (1965)
eB/De	--	--	90	59	Murphy (1966)
f/He	--	--	--	17	
HeO	323	--	32	-	Akamatsu et al. (1967)

*m = malignant tumors.

TABLE 4. SPONTANEOUS HEPATIC TUMORS IN VARIOUS STRAINS AND SUBSTRAINS OF MICE

<u>Substrain</u>	<u>Numbers</u>		<u>Incidence %</u>		<u>References</u>
	<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>	
STS	--	18	--	0	Miller et al. (1964)
DBA/2eBDe	--	--	--	5	Murphy (1966)
HR/De	--	--	12	6	
Wild Mice	--	--	9	3	
CBA/Cb/Se	37	47	11	4	Severi & Biancifiore (1968)
CBA	710	710	0	0	Kirby (1945)
CBA	285	229	41	27	Pybus & Miller (1942)
ICR	28	--	0	-	Takayama (1969)
RF	262	--	3	-	Clapp et al. (1971)

Table 5 lists data on chemically induced lesions in the liver in various strains and substrains of mice. Table 6 indicates the vagaries of mice where the same strain differs in incidences of hepatoma with time.

TABLE 5. CHEMICALLY INDUCED HEPATIC TUMORS IN MICE
DIMETHYLNITROSAMINE

Treatment	Treatment Duration Days	Duration of Experiment Days	Strain	Numbers	Tumors %		References
					Hepatocellular	Endothelial	
50 ppm	150		ddN	210	0	10m	Takayama & Oota (1965)
DW or D	150-300		ICR	340	6	20m	
	150	To death	C ₃ H	250	24b	0	
100 ppm		Average					
DW or D	150	225-330	ddN	170	0	23m	
200 ppm							
DW or D	150		ICR	110	0	0	
0.001%	141	To death	BALB/c	410	0	19b	Toth et al. (1964)
DW		70-360 (max)		460	0	19b	
0/19mM/kg		365	CFW/D	420	0	0	Frei (1970)
i.p. once							
0.91 mg/kg	267-	Mean survi-	RF	940	2	96m	Clapp et al. (1971)
Daily DW	mean	val time - 360					
1.7 mg/kg		285					
Daily DW	180	(age)	BALB/c	150	0	20m	

D = diet, DW = distilled water, b = benign, and m = malignant

TABLE 6. CHANGING INCIDENCE WITH TIME OF HEPATOMA IN MICE
(H. B. Andervont, 1950)

Series	Strain	Sex	Numbers	Tumors %	Year
1	C ₃ H	F	362	11	1942
2	C ₃ H	F	179	5	1943/5
3	C ₃ H	M	43	12	1943
4	C ₃ H	M	86	55	1944
7	CBA	M	68	29	1947
7	CBA	M	40	20	to
7	CBA	F	63	5	1948
7	CBA	F	29	3	

These data taken alone might not cause concern when using mice for safety testing. Taken together, however, and knowing that for some undetermined reason most chemicals appear to affect more often the liver of mice, the interpretation of the data is difficult if not impossible when there is already extant a significant but variable background incidence of liver lesions.

EXTRAPOLATION OF ANIMAL DATA TO MAN

Attempts to extrapolate animal data to man are fraught with many hazards, particularly if the data available may be used in support of some Federal agency regulatory action. The scientific community is a heterogeneous group of individuals with sincere but diverse opinions, particularly in regard to carcinogenesis and safety testing. We seem to be polarized in our views regarding the role of chemicals in this phenomenon. At one extreme are those individuals who feel that the only proved carcinogens are those which have been shown to be suspect by acceptable epidemiologic studies in man. At the other extreme are those who adopt the simplistic view that a suggestive carcinogenic response in any species, at any dose, is absolute proof that the compound in question is a potential danger to man and should be immediately banned from the environment. One example of this philosophy is embodied in the so-called Delaney clause in the Food, Drug and Cosmetic Act, which initially was intended to be applied to food additives and other kinds of materials that might find their way into the food chain and be inadvertently ingested, or in which some other accidental exposure might occur. The rule is simply this: any carcinogenic response, in any species, at any dose, immediately eliminates a compound from any further consideration. Thus, in the case of carcinogenesis, as opposed to other types of toxicologic investigations, the "all or nothing" rule applies. The dose response concept is thus largely ignored, a situation that tends to violate the basic tenets of toxicology. Although such an interpretation has not yet been applied to drugs, it seems to be gradually gaining wider acceptance in various academic and governmental areas.

In developing information upon which to assign levels of "relative safety" or "risk factors", a statistical formula such as that proposed by Mantel and Byran (1961) might be considered. These authors suggest that some estimate of a dose of a given agent can be devised which could be considered "virtually safe." This assumes, of course, that one first defines some arbitrary level of permissible risk, no matter how small, rather than insisting on absolute safety which is, of course, unobtainable. The point of interest is that since direct observations cannot be made which establish unequivocally that the risk at some dose is clearly low, indirect conservative procedures for the determination of low risk levels become necessary.

Mantel and Byran (1961) state, "Absolute safety can never be unquestionably demonstrated experimentally. Rather, experimental results can be used only to establish limits on the risk involved. With the specification of some level of risk, no matter how small, the possibility of determining whether or not that risk is exceeded opens. We may, for example, assume that a risk of 1/100 million is so low as to constitute 'virtual safety.' Other arbitrary definitions of 'virtual safety' may be employed as conditions require."

"In principle, one could use an experimental protocol sufficiently large to demonstrate that 'virtual safety' obtained. For this purpose it must be realized that an observed outcome of no tumors among 100 million treated mice does not necessarily demonstrate clearly that treatment was either absolutely or virtually safe. This outcome could arise with a probability that the risk was under 1 percent, even if the risk involved were as high as 4.6/100 million. It would in fact require a total of some 460 million tumor-free mice to demonstrate at the 99 percent assurance level that 'virtual safety' obtained. Similarly, tumor-free results for 10,000 mice would only indicate that the risk was less than 1/2,200 and it would require tumor-free results in a total of some 450 mice to establish with high probability that the risk was under 1 percent."

Such astronomical figures required for direct observation tend to stagger the imagination. However, the statistical formula devised by these investigators is based on the assumption that the relationship observed between tumor occurrence and dose at the levels tested will continue to apply to the regions to which extrapolation is being made, in which situation studies of feasible size can be used. It is apparent, of course, that the first requirement for such a statistical formula is that the carcinogenic dose be defined, and that the dose response concept be accepted for carcinogenicity evaluation as it is for other types of toxicologic investigations. I think we can agree that in the present state of the art the use of the mouse for such purposes would pose a considerable problem. In the absence of other workable systems, however, such an approach might be considered in arriving at decisions regarding "relative safety" or risk factors.

Another series of reports developed by the National Institute of Occupational Safety and Health (NIOSH) points out some interesting facets of using experimental animals for carcinogenic assessment of chemicals. The available data on 14 compounds considered to be known or suspected carcinogens in man were screened by scientists of the National Institute of Occupational Safety and Health (NIOSH). The purpose of the review was to make recommendations regarding the handling and use of these substances in industrial and research settings. Since this exercise represents an attempt on the part of knowledgeable individuals to extrapolate experimental data to man, and cites the basis for conclusions that were drawn, selected examples of the reports were extracted and used to illustrate comparative response to compounds considered to be carcinogenic for man. A review of the following tables will be of interest.

Table 7 compares rat and man; Table 8, mouse and man; Table 9, hamster and man; Table 10, dog and man; and Table 11, monkey and man. Without attempting to explain the impossible, we can say that in most cases there are similarities (i.e., Table 7, beta-naphthylamine and bladder tumors in rat and man) and cases where the animal species and man are dissimilar (benzidine). For rat and man, in terms of target organs, they were similar

in response in two cases, dissimilar in three. Man and mouse were similar in three, dissimilar in three. Data were not sufficient to judge the hamster (Table 9) but the dog and man were similar in the three compounds where comparative data were available (Table 10). Interestingly, the monkey and man were similar in one case and dissimilar in the other but only two compounds could be compared (Table 11).

TABLE 7. A COMPARISON OF RAT AND MAN IN RESPONSE TO KNOWN OR SUSPECTED HUMAN CARCINOGENS

Compound	Species	Route	Dose/Duration	Target Organ(s)	Comment
Benzidine	Rat	sc	15 mg/wk (lifetime)	Liver (M)	Tumors in males only
		sc	300 mg/6 mos	Liver Ear Injection site	
		oral	5 mg x 10 (q 3 days)	Mammary gland	
		vapor	27 mg/20 mos	WBC (Leukemia) Mammary gland Liver	Tumors in males and females
	Man	N.S. *	Occupational exposure	Bladder Kidney Pancreas	Interpretation complicated by concomitant exposure to other known carcinogens
Beta-Naphthylamine	Rat	oral	0.067% in diet	Bladder Liver Lung	
		ip	100 mg/kg/wk	Abdomen (site) Salivary gland	
	Man	Occupational exposure	Unknown	Bladder	
4-Aminodiphenyl	Rat	sc	N.S. *	Liver Intestine Mammary gland	
	Man	Occupational exposure	Unknown	Bladder	High incidence of bladder tumors
Bis(chloromethyl) ether	Rat	parenteral	9 mg/mo (9 mo)	Injection site	
		vapor	0.1 ppm (101 exposures)	Lung Nose	
	Man	vapor	Unknown	Lung	Increased incidence of oat cell carcinoma
Chloromethyl methyl ether	Rat	sc	1-3 mg (3-4 times/mo)	Injection site	Palpable lesions - not defined as malignancies
		topical	1000 & 100 µg (duration N.S.)*	Application site	Possible "irritating agent" (phorbol ester promoting agent)
		sc	3 mg (duration N.S.)*	Injection site	
	Man	Occupational exposure	Unknown (1-14 years)	Lung	High incidence oat cell carcinoma. Note: CMME hydrolyzed to MeOH + HCOH + HCl which recombines to BCME spontaneously.

*Not Stated. (Data excerpted from NIOSH Report, July, 1973)

TABLE 8. A COMPARISON OF MOUSE AND MAN IN RESPONSE TO KNOWN OR SUSPECTED HUMAN CARCINOGENS

<u>Compound</u>	<u>Species</u>	<u>Route</u>	<u>Dose/Duration</u>	<u>Target Organ(s)</u>	<u>Comment</u>
Benzidine	Mouse	sc	6 mg/wk 15-28 mo	Liver	
	Man	N.S.*	Occupational exposure	Bladder Kidney Pancreas	Interpretation complicated by concomitant exposure to other known carcinogens
Beta-Naphthylamine	Mouse	sc	3 g/50 wk	Liver	
	Man	Occupational exposure	Unknown	Bladder	
Alpha-Naphthylamine	Mouse	Drinking water	100 mg/l (duration N.S.)*	None reported	
		oral	250 mg (single dose)	None reported	
	Man	Occupational exposure	(duration N.S.)*	Bladder	
4-Aminodiphenyl	Mouse	oral	38 mg/9 mo (2 x wk)	Bladder	
		oral	1.5 mg/wk (x 5 wk)	Liver Bladder	
		sc	200 mg (single dose)	Liver	Increased incidence of hepatomas; 19/20 males; 4/23 females
	Man	Occupational exposure	Unknown	Bladder	High incidence of bladder tumors
Bis(chloromethyl) ether	Mouse	topical	6 mg/wk (50 wk)	Application site	
		sc	12.5 ml/kg (single dose)	Lung	
		vapor	1 ppm (82 days)	Lung	Only slightly higher tumor incidence in treated over control
	Man	vapor	Unknown	Lung	Increased incidence of oat cell carcinoma
Chloromethyl methyl ether	Mouse	topical	6 mg/wk (42 wk)	See comment	Incomplete carcinogen
		sc	125 ml/kg (single)	See comment	Some BCME contaminant
		vapor	2 ppm (10 exposures)	Lung	Carcinogenicity not definitely established because of contamination with BCME
		sc	300 µg/wk (26 wk)	Injection site	
	Man	Occupational exposure	Unknown (1-14 years)	Lung	High incidence of oat cell carcinoma. Note: CMME hydrolyzed to MeOH + HCOH + HCl which recombines to BCME spontaneously.

*Not Stated. (Data excerpted from NIOSH Report, July, 1973)

TABLE 9. A COMPARISON OF HAMSTER AND MAN IN RESPONSE TO KNOWN OR SUSPECTED HUMAN CARCINOGENS

<u>Compound</u>	<u>Species</u>	<u>Route</u>	<u>Dose/Duration</u>	<u>Target Organ(s)</u>	<u>Comment</u>
Benzidine	Hamster	diet	0.1% lifetime	Liver Biliary system	
	Man	N.S.*	Occupational exposure	Bladder Kidney Pancreas	Interpretation complicated by concomitant exposure to other known carcinogens
Beta-Naphthylamine	Hamster	oral	1% in diet	Bladder	
	Man	Occupational exposure	Unknown	Bladder	

*Not Stated. (Data excerpted from NIOSH Report, July, 1973)

TABLE 10. A COMPARISON OF DOG AND MAN IN RESPONSE TO KNOWN OR SUSPECTED HUMAN CARCINOGENS

<u>Compound</u>	<u>Species</u>	<u>Route</u>	<u>Dose/Duration</u>	<u>Target Organ(s)</u>	<u>Comment</u>
Benzidine	Dog	diet	325 g in 5 yrs	Bladder	1 carcinoma in 7 dogs
	Man	N.S.*	Occupational exposure	Bladder Kidney Pancreas	Interpretation complicated by concomitant exposure to other known carcinogens
Beta-Naphthylamine	Dog	sc and diet	Variable	Bladder	
		oral	310 g total	Bladder	
		oral	1000-2000 mg/wk	Bladder	
	Man	Occupational exposure	Unknown	Bladder	
4-Aminodiphenyl	Dog	oral	Variable	Bladder	
		oral	0.9 g/wk (34 mo)	Bladder	
		oral	0.5 g/wk (3 yr)	Bladder	
		oral	50 mg/kg (single dose)	---	No tumors during 5 years observation
	Man	Occupational exposure	Unknown	Bladder	High incidence of bladder tumors

*Not Stated. (Data excerpted from NIOSH Report, July, 1973)

TABLE 11. A COMPARISON OF MONKEY AND MAN IN RESPONSE TO KNOWN OR SUSPECTED HUMAN CARCINOGENS

<u>Compound</u>	<u>Species</u>	<u>Route</u>	<u>Dose/Duration</u>	<u>Target Organ(s)</u>	<u>Comment</u>
Benzidine	Monkey	sc	50-200 mg/wk (duration N.S.)*	---	No carcinogenic effect
	Man	N.S.*	Occupational exposure	Bladder Kidney Pancreas	Interpretation complicated by concomitant exposure to other known carcinogens
Beta-Naphthylamine	Monkey	oral	6.25-400 mg/kg	Bladder	
	Man	Occupational exposure	Unknown	Bladder	

*Not Stated. (Data excerpted from NIOSH Report, July, 1973)

Thus, we can predict with confidence only to a very limited extent from one species to another relative to a response to a given chemical. A number of factors must be taken into account in choosing a species or strain and careful consideration must be given to target organ of interest, metabolism where known, etc. Even with all new methodology at our disposal for assessing potential dangers, experimental animals remain our best, most reliable tools and, except for the mouse, data gained from the use of the various species and strains provide a reasonable basis for safety testing of environmental chemicals.

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STATISTICAL MODELS FOR ESTIMATING CARCINOGENIC RISKS FROM ANIMAL DATA

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INTRODUCTION

A problem of great concern currently faced by regulatory agencies is the prediction of carcinogenic risk of various environmental agents to man. Typically, a prediction of carcinogenic incidence in humans is required at a level of exposure considerably below experimental animal levels from which the predictions are derived. This then, in its simplest form, presents two problems; first, the statistical extrapolation to low-dose levels, and secondly, the mouse-to-man or species extrapolation. Although the methodology is currently considered to be inadequate in both instances, the statistical techniques used for low-dose extrapolation will be briefly discussed in this paper, the rationale being that this area has received considerably more attention from statisticians than species extrapolation.

DOSE RESPONSE MODELS

In order to predict a response outside the experimental range, a functional relationship between dose and response is needed. This relationship is usually expressed by a parametric model which describes the distribution of response for a given set of experimental conditions, including dose level. These models are completely specified except for a few unknown parameters which are either estimated from specific experimental data which the model attempts to describe, or by appealing empirically to the results of large classes of similar experiments. It is possible to have mathematical models which have had a history of adequately fitting certain types of experimental data, but which have little biological basis or justification.

The mathematical models used for relating toxic response and dose levels in animal survival studies fall basically into two categories. Most common are models which deal with a dichotomous response. That is, the response is either that a particular condition is present or that it is not. Examples are the one-hit model (Gross et al., 1970) and the use of the probit

model (Mantel and Bryan, 1961) for evaluating levels of toxicity. The second category consists of models which deal with the distribution of the "time-to-occurrence" and its relationship of dose level. The occurrence may, for a given situation, be the appearance or detection of a tumor. In another situation, the occurrence may be death due to a particular disease. The parametric models most often considered for representing this time distribution are the log normal and the Weibull.

The low dose extrapolation problem is concerned with determining the dose that will produce a response which is, at most, a given preassigned level. Given a model and a set of data, this dose can be directly estimated. Further, a dose can be estimated which will be below the desired unknown dose with a given level of confidence. The difficulty with this approach, however, is that it is highly dependent on the assumption of a particular model.

A second approach to the extrapolation problem due to Mantel and Bryan (1961) is to select a mathematical model which, with high confidence, will provide an upper bound to the true unknown response curve. The remaining parameters for this model are then estimated from data and the estimated dose produced. It must be emphasized that this dose is not the best estimate of the unknown dose associated with the desired response. It is, instead, a dose which is most likely to be below the dose required to give the desired response. The justification for this more conservative approach lies in the fact that, since very little is known about the lower ends of a dose response curve, the choice of the model becomes critical in that very region of the curve. Basically, the problem is that, in the experimental region of the curve, several competing models will appear to fit the data well, while the tails of these response distribution curves often differ by many orders of magnitude (see FDA Advisory Committee, 1971). Therefore, until there is a better understanding of the biological mechanisms involved, it will be difficult to choose properly a model based solely on experimental data.

INCIDENCE MODELS

Incidence or dichotomous response models which relate dose to the probability of a particular condition (e.g. carcinogenesis) being present are the most common in extrapolation. These models are usually of the form

$$p_d = F(\alpha + \beta \log d)$$

where p_d is the probability of response at dose d , F is any cumulative distribution and α , β are unknown parameters whose values must be determined. If, for example, F is the cumulative normal distribution, then the model states that the probit of the probability of response is linear in log-dose. Other common choices of F are the logistic distribution and the Weibull or

extreme value distribution. These models can be explicitly expressed as

$$\text{Probit: } p_d = \Phi(\alpha + \beta \log d)$$

$$\text{Logistic: } p_d = [\exp(\alpha + \beta \log d) + 1]^{-1}$$

$$\text{Extreme value: } p_d = 1 - \exp[\exp(\alpha + \beta \log d)]$$

where Φ denotes the cumulative normal distribution. It should be noted that when $\beta = 1$ in the extreme value model, one had

$$p_d = 1 - \exp\{-\alpha d\}$$

which is recognized as the one-hit model. Further, for small values of αd (i. e. low dose) the one-hit model is essentially numerically identical to the linear model

$$p_d = \alpha d.$$

For each of these models, incidence data may be used to directly estimate the unknown parameters α and β . Furthermore, a dose estimate can be constructed which, at any prescribed confidence level, will be less than or equal to the unknown dose level associated with any particular desired response level. This is a standard statistical approach to the problem. The difficulty is that this approach assumes the unknown dose-response curve is functionally the one used in the estimation procedure. Although this is not critical for interpolation problems, it is an extremely critical assumption for extrapolation some distance from the experimental range.

Mantel and Bryan (1961) attempted to circumvent this difficulty by considering the probit model for setting "virtually safe" dose levels. They observed that the slope β is generally greater than one in animal carcinogenesis studies. Thus, as a conservative lower bound, they set the slope equal to one and then used an upper 99% confidence interval for the observed incidence for the extrapolation. This technique provides a dose level which is, with at least 99% confidence, less than the desired dose level. This is correct, providing the true dose-response curve is bounded above by the probit curve with $\beta = 1$. The technique can, in theory, be applied equally well with the logistic and the extreme-value models. The empirical observation that the parameter β is at least a certain value must also be made for these two models.

The choice of an appropriate model for the purpose of extrapolation is particularly difficult since all are only approximations of the true state of nature. In the typical experimental range these dose-response models appear to be essentially identical, as shown by the FDA Advisory Committee on Protocols for Safety Evaluation (1971). On the other hand, the FDA Committee

also illustrated that there are great differences in these models at the low dose levels. One may therefore conclude that it is unlikely that a proper model can be chosen from experimental incidence data, yet the choice of one model over another makes important differences in the estimated dose for a low response level. Even if the proper model could be chosen from the incidence data, there is the possibility that one or more unknown parameters may be functions of dose. For example, the slope parameter β in the probit model could possibly decrease with decreasing dose level in a given experimental situation. With the direct application of the Mantel-Bryan technique there is the additional difficulty of occasionally obtaining a value of the slope β less than one in the experimental design.

Therefore, from a public health standpoint, one then is compelled to select an extrapolation model which is not likely to grossly underestimate the risk at the low dose levels. This choice would be the linear model for basically two reasons. The first being that the dose response curve is quite likely to be concave in the low dose range and hence a linear dose response would provide an upper bound to the predicted risk. The second reason is based on the fact that very general multistage models with background incidences are approximately linear in the low dose range. So that the linear model may actually be a very good estimate instead of simply an upper bound for risks at low doses.

TIME-TO-OCCURRENCE MODELS

The second group of models relating response and dose measure time-to-occurrence of the response instead of simple incidence. Time-to-occurrence for a given problem will typically be defined as death due to a particular disease, or possibly the time of first appearance of the disease. This information is often available for both animal survival studies and human data and has proved to be quite useful in interpreting some experimental results (e.g., Hoel and Walburg, 1972).

On the basis of experimental and theoretical considerations, two distributions of time-to-occurrence of cancer have received particular attention. Blum (1959) and Druckrey (1967) have proposed a log normal distribution with median time-to-cancer depending on dose, but with standard deviation independent of the dose. The Weibull distribution has been suggested by human cancer incidence patterns (see Cook, Doll and Fellingham, 1969; Lee and O'Neill, 1971) and has been predicted by some theoretical models of carcinogenesis (Pike, 1966).

Albert and Altshuler (1973) have made a study of the Blum-Druckrey model and have applied it to a variety of carcinogenesis data sets. They specifically assume that if cancer were the only cause of death, those deaths

would have a log normal distribution. They further relate a dose level d to the distribution by assuming $t^n d = C$ where t is the median single-risk time of appearance of cancer, n is a parameter assumed to be greater than one and C is a constant. Since n is greater than one, the median time-to-cancer decreases with increasing dose for this model. The standard deviation of the log normal distribution is assumed to be constant for varying dose levels.

A number of investigators (Peto, Lee and Paige, 1972; Peto and Lee, 1973; and Day, 1967) have used the Weibull distribution for a time-to-tumor distribution. The Weibull model, which Peto, Lee and Paige (1972) consider, defines the incidence rate of primary tumors at a particular site at age t as equal to $b(t-w)^k$. The constant of proportionality b depends on the dose while the parameters k, w do not. Also, these authors interpret the parameter k as the number of distinct "stages" which are involved in the development of a tumor with w representing the latent period. In order to bring dose dependency into the model, the parameter b , which depends on the dose d , would be replaced by cd^m where c and m are not dose dependent.

In comparing the two models, Peto, Lee and Paige (1972) have attempted to fit both the log normal and Weibull distribution to a large number of experimental data groups. They found that the Weibull distribution agreed with the data considerably better than did the log normal. They further assert that the Weibull is preferable because of the theoretical basis given by Pike (1966) and the observation of Gehan (1969) that log normal distribution does not have a monotone hazard function. Albert and Altshuler (1973) have also attempted to fit the log normal distribution to a variety of data sets. They maintain that the proper model depends on what type of cancer is being considered. No mathematical model is likely to correctly describe a time distribution for a given cancer and the dependence of such a distribution upon dose for extrapolation purposes. It is therefore of practical value to have available several reasonable models which collectively could be applied to a given problem.

With both log normal and Weibull models there are considerable technical problems in the actual estimation of the unknown parameters. These become particularly complicated in the face of competing risks. If these statistical difficulties can be satisfactorily overcome, extrapolation could then be considered. Again, as expected, low dose extrapolation is highly dependent on the choice of which of the two models to apply. One set of calculations in Chand and Hoel (1974) gave differing values for the probability of tumor occurrence in a particular hypothetical experimental situation. At a given low dose it was found that the log normal model predicted an incidence of 2×10^{-7} , while the Weibull model yielded, at the same dose, an incidence of 2.4×10^{-4} . The magnitude of the difference increased as the dose level decreased. This is much the same as the situation with the

dichotomous response model. Thus it may be concluded that the problems of model dependency in low dose extrapolations will be present in both the dichotomous and time-to-occurrence situations.

DISCUSSION

All of the models discussed are only approximations with little or no mechanistic justification. The only exception possibly may be the linear relationship in the low-dose region. Thus it is not surprising that extrapolation to extremely low incidence levels is highly model dependent and for typical sized experiments leads to very low dose levels. If the extrapolation techniques were applied to data which shows no significant carcinogenic effect, one also finds very low estimated dose levels associated with low risk levels. This then presents the practical problem of how to determine dose levels of agents which have not been shown to be positive by carcinogenic testing. The solution probably is in extrapolation to predicted incidence levels which are higher than the typical 10^{-6} to 10^{-8} used for carcinogens. The reason why extrapolation is needed is to incorporate information such as sample size and statistical confidence into setting usage levels of the test compound.

Finally, it should be mentioned that there are many unsolved statistical and biological problems in extrapolation besides model selection. Some of these are combining data from several dose levels and experiments, adjustments for background and incorporation of data from various strains and species.

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RELEVANCE OF MUTAGENESIS TO CARCINOGENESIS

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CORRELATION BETWEEN CARCINOGENICITY AND MUTAGENICITY

The implication of a mutation event as one of the prerequisites for induction of tumors dates back to a period substantially earlier than the formation of the somatic mutation theory of cancer in 1928, (Bauer 1928). The heritable changes produced in the phenotype in both carcinogenesis and mutagenesis superficially made the somatic theory of cancer attractive. Many alternatives to the somatic theory of carcinogenesis can be found in the literature with one of the major theories relating to viral oncogenesis. According to this concept the chemical carcinogen activates a latent virus to induce neoplasia. This theory is not in apparent conflict with the somatic theory, as in both cases we are dealing with an altered genome.

On the basis of available information, Burdette in 1955 concluded that somatic point mutation cannot be regarded as a general mechanism of cancer initiation. In the intervening years, with an understanding of the molecular basis of mutation and by the evaluation of compounds for carcinogenicity and mutagenicity in comparable systems, the relationship between mutagens and carcinogens has become clear. In a recent survey of the literature, Miller (1970) concluded that many and perhaps all carcinogens are mutagens and that many, but possibly not all, mutagens are carcinogens (Miller, 1970).

As recently discussed by Von Lanker (1974) the most likely sequence of events in the carcinogenic process would be the linking of the carcinogen to DNA bases with the partial excision of the altered bases. The final alteration of DNA could be due to mispairing during the repair process or because the repair process would not be complete. Unrepaired DNA would then either not be transmissible or the transcription may be erroneous. The phenotypic changes that constitute a malignant cell would then occur from a combination of point mutations or deletions which would confer under certain conditions a selective advantage for the altered malignant cell to multiply.

The practical demonstration of the correlation between genetic alteration (mutation) and oncogenetics could not occur until methodology, principally in animal systems, for detecting mutagenic activity become available.

Our ability to develop the present strong correlation between mutagenic agents and carcinogenic compounds is a direct consequence of our ability to determine both effects in mammalian systems. Prior to the development of mammalian tests for mutagenicity, mutagenic agents were evaluated for carcinogenicity in rodents. The major exceptions that were previously found for carcinogens not being mutagenic have been for the most part resolved by the use of rodents for testing both types of activity. Carcinogenic compounds such as cycasin and nitrosamines, which are not mutagenic when tested directly on bacteria, are now known to be mutagenic as well as carcinogenic when the active metabolites are evaluated (Legator, 1973). Procedures such as the host-mediated assay have contributed greatly to the establishment of a qualitative correlation between carcinogenic and mutagenic compounds. The use of available mammalian tests for mutagenicity should identify carcinogenic compounds in a fraction of the time needed for the conventional carcinogenic assay.

DETECTION AND CHARACTERIZATION OF CHEMICAL MUTAGENS

In a discussion on methodology a clear distinction should be made between detection of chemical mutagens in a screening program and characterization of the genetic lesion induced by the chemical. The most appropriate systems for characterizing genetic lesions are those in which the genetics of the system have been extensively studied and in this category one would place a variety of microbial systems as well as Drosophila.

In the initial screen for mutagenic activity the lesions need not be known, but a comprehensive initial screen should detect the full spectrum of genetic alterations. Table 1 describes the capabilities of the specific test in terms of the genetic lesion that can be detected. In an in-depth study of chemical mutagens one would first select those test systems that have the capability of indicating the various type of DNA alterations produced by chemicals that are active per se or those which are activated by enzymes of the tissues or intestinal microbial flora of the host.

TABLE 1. GENETIC LESIONS DETECTED BY VARIOUS SYSTEMS^(a)

Mutations				Chromosomal Aberrations				Test Systems for Detecting Metabolites
	Forward and/or Reverse	Specific Loci (Multiple)	Recombination	Dominant Lethal	Translocation	Deletions and Duplications	Non - disjunction	
Systems								
1. <u>Microbial</u>								
a. <u>Procaryote</u>								
1. S. typhimurium	x							x
2. E. coli	x							x
b. <u>Fungal</u>								
1. Neurospora	x					x		
2. Aspergillis	x	x	x				x	x
3. Yeast	x	x	x	x			x	x
2. <u>Plant</u>								
a. <u>Vicia</u>								
					x	x	x	
b. Tradescantia	x				x	x	x	
3. <u>Insects</u>								
a. <u>Drosophila</u>								
	x	x	x	x	x	x	x	x
b. Habrobracon	x	x	x	x				
c. Bombyx	x	x						
4. <u>In Vitro</u>								
<u>Mammalian Cell Systems</u>								
a. Chinese Hamster	x				x	x	x	x
b. Mouse Lymphoma	x				x	x	x	x
5. <u>In Vitro</u>								
<u>Mammalian Systems</u>								
a. Mouse		x		x	x	x	x	
b. Rat				x	x	x	x	
6. <u>Man</u>								
					x	x	x	

(a) Detailed description of test systems can be found in *Chemical Mutagens*, Volume 1-3, A. Hollaender, Editor, Plenum Press, N. Y. 1971 and 1973.

METHODOLOGIES IN MUTAGENICITY TESTING

The available methods can be conveniently classified as those that are capable of detecting (a) direct acting and (b) indirect acting mutagens. In turn, systems with the capability of detecting indirect acting compounds are (a) those possessing the appropriate metabolic machinery to convert inactive substances to mutagenic forms, and (b) those not possessing this machinery but characterized by special genetic properties so that they serve as "indicator organisms" evaluating products produced in the intact host or in various organs thereof for mutagenic activity. These procedures are illustrated in Table 2. Detailed discussions of all of these methods can be found in the three volumes of Chemical Mutagens edited by A. Hollaender (1971-1973) and in a method manual currently in preparation (Kilbey et al., in press).

TABLE 2. DETECTION METHODS BASED ON CHEMICAL ACTIVITY

	<u>Procedure</u>	<u>Type of Chemical Detected</u>
A.	In Vitro Systems i.e. phage, bacteria, yeast	Direct acting chemicals not requiring host metabolism
B.	Microsomal Activating System, in vitro	Selected compounds which can be activated by the in vitro microsomal techniques
C.	Detection of host metabolites by indirect indicators i.e. host-mediated assay	Direct acting chemicals as well as metabolites formed by a variety of routes in intact animal, specifically for chemicals that induce point mutations when microbial indicators are used
D.	Drosophila	Chemicals metabolized in Drosophila in a manner similar to experimental animals
E.	Studies in intact animals, i.e. repair, cytogenetic, dominant lethal, and mouse specific locus test	Potentially all mutagenic agents, however with the exception of repair and specific locus test only chromosome breaking agents detected

TESTS THAT CAN DETECT DIRECT ACTING COMPOUNDS, I. E.,
COMPOUNDS NOT REQUIRING METABOLIC ACTIVATION

Probably the most useful procedures for rapid screening purposes are microorganisms, and mammalian in vitro cell culture systems. The large population that can easily be raised, short generation time, simplicity and low cost are characteristic of the majority of these testing procedures. In mutation research, we are interested in the alterations of a specific chemical, DNA; therefore, we are dealing with a chemical where the nature of the material in terms of reactivity, stability and the mode of protein formation are essentially the same at all levels of biological organization. Since DNA and the types of genetic alterations inducible therein are similar in all organisms, credence is given to procedures in all test systems for characterizing known active compounds. On the other hand, microbial systems suffer in their inability to detect most mutagenic substances that may be active in man. Cellular uptake and distribution, metabolism, detoxification dosage and method of administration of the chemical are among many factors that can profoundly effect the mutagenic process. Mammalian cells in cultures are sufficiently removed from man and intact mammals to also be of limited value for detecting all but direct acting compounds.

A wide range of microorganisms is available for characterizing chemical mutagens including *Neurospora*, various strain of yeast, bacteria. The tester strains of *Salmonella typhimurium* developed by B. Ames (1971) is probably the best developed and the most widely used microbial system in this field. These tester strains are mutants where the genetic alteration is known (base pair substitutions or frameshift mutations), and can be used to detect back mutations (reversion to prototrophy). The sensitivity of these tester strains has been markedly enhanced by the introduction of a deletion of one of the genes of the excision repair system and a mutant which is deficient in the lipopolysaccharide which coats the surface of these bacteria. The repair deficient mutant greatly increases the sensitivity of the system and the loss of the polysaccharide coat removes a barrier for the penetration of lipophilic chemicals. It has recently been shown that frameshift mutagens have a great deal of specificity as to the repetitive DNA sequence in which they are active and tester strains have been and are being developed with known "hot spots." Although several tester strains have to be used in characterizing mutagenic agents by this system, the level of sophistication achieved with *Salmonella typhimurium* probably makes this one of the best available microbial tests for the characterization of chemical mutagens.

IN VITRO MICROSOMAL ENZYME STUDIES

Those compounds that are metabolized by liver-microsomal agents can be conveniently detected by the use of liver microsomal hydroxylase systems (Popper et al., 1973). Some aromatic amines, polycyclic hydrocarbons and naturally occurring chemicals such as aflatoxin (Garner et al., 1972 and Ames et al., 1973) can be detected in this system. The frequently coupled indicator organism with the microsomal activation procedure has been S. typhimurium but almost any indicator including other microorganisms; additionally, mammalian cells or indeed Drosophila could be used in conjunction with microsomal activation. Whereas this activation system is of distinct advantage in characterizing specific classes of chemical mutagens, it can not reflect either quantitatively or qualitatively the dynamic processes that occur in an intact host. Even if this in vitro procedure could detect all metabolic products produced by microsomal enzymes, which it obviously can not, metabolites produced by other routes would be overlooked. Compounds such as cycasin (Gabridge et al., 1969) which are activated by intestinal flora, the azo dyes (Legator, in press) and chloropurine (Ray and Holden, 1974) are examples of mutagenic agents that could not be detected by microsomal activation methods. The simplicity and relative inexpensiveness of this procedure are appealing but it should be recognized this method can only detect a limited spectrum of active compounds.

SCREENING, BY INDIRECT INDICATOR, FOR MUTAGENIC COMPOUNDS
PRODUCED IN THE INTACT ANIMAL

These procedures utilize the intact animal to either activate (produce a mutagenic metabolite) or to indicate the elimination of mutagenic activity by the production of non-mutagenic metabolites. Bacteria are the most widely used indicator organisms, where screening for point mutations is the commonly employed procedure. Additionally, a variety of other microorganisms as well as mammalian cells can be used.

The Host-Mediated Assay - This assay was introduced in 1969 (Gabridge and Legator, 1969), and the various modifications since its initial inception has made this indirect method for detecting mutations a primary tool for characterizing mutagenic agents. In this assay, the animal during and after treatment with a potential chemical mutagen is injected with the indicator organisms in which mutation frequency can be measured. After a sufficient time period, the indicator organism is withdrawn from the animal and the induction of mutants is determined. The comparison between the mutagenic action of the compound on the indicator directly and in the host-mediated assay indicates whether (a) the host can detoxify the compound or (b) mutagenic products can be found as a result of host metabolism.

The majority of studies carried out with this procedure have relied on the addition of the indicator to the intraperitoneal cavity of the treated animal. Several modifications of this method have attempted to detect mutagenic activity in various organs and tissues of the animal (Fiscor and Muthian, 1971; Mohn and Ellenberger, 1973).

Analysis of Blood and Urine - In a series of initial papers on the host-mediated assay (Gabridge et al., 1969; Marquardt and Siebert, 1971) the analysis of blood and urine in animals treated with Streptozotocin or cytoxan revealed the presence of mutagenic activity. This procedure can be extremely useful especially if information is available as to the pharmacokinetics of the compound. A variety of indicators can be used in this procedure, several samples can be tested and, of special importance, this technique can be used to detect mutagenic substances in man. In urine analysis the use of specific enzymes to disassociate various conjugates has been described and method of concentrating both blood and urine prior to the evaluation of mutagenic substances has been reported (Durstun and Ames, 1974; Legator et al., 1974). Although these procedures can potentially detect active compounds regardless of where they are produced, these methods can not conclusively demonstrate that the compound can induce mutations in animals. The possible repair of the genetic lesion by the host can not be ascertained by these procedures.

STUDIES CONDUCTED DIRECTLY IN THE INTACT ANIMAL

The procedures in animals are still limited. Methodologies exist for characterizing chromosomal aberrations but no definitive procedures exist for detecting point mutations with the possible exception of the specific locus test. Chromosomal analysis, dominant lethal test, and translocation studies are all basically methods to determine chromosome alterations. In addition to cytogenetic alterations, the induction of genetic repair by chemicals altering DNA can also be determined in both somatic and germinal cells.

In Vivo Cytogenetic Studies - Cytogenetic studies can be carried out in almost all biological species including man. It is the only procedure where the results of genetic damage can be directly observed through the light microscope. In animals, somatic as well as germ cells can be analyzed, and when carried out in vivo it is a meaningful selective assay. Chromosome alterations may be subdivided into three main categories: (a) numerical changes, (b) structural changes, recognizable at mitosis, most of which involve the formation of fragments, and (c) structural changes, recognizable during meiosis. The procedures for recognizing these alterations and a discussion on the utilization of cytogenetic analysis in a toxicological screen were reported by an

Ad Hoc Committee of the Environmental Mutagen Society (Nichols et al., 1972). A procedure for examining chromosomes in vivo at anaphase rather than metaphase has been described (Palmer et al., 1972). In this procedure, the animals are pretreated with colcemide (not colchicine) to collect mitotic figures, releasing the colcemide block, and allowing the mitotic cells to progress to anaphase (approximately 1-1/2 hours). The potential rapidity of this method should greatly facilitate the screening of potential mutagenic agents and further enhance the utility of cytogenetic screening for genetic damage.

Of special interest is the micronuclei procedure for evaluating chemically induced chromosomal abnormalities (Matter and Schmid, 1971; Schmid, 1972). The principle behind this procedure is that mitotic cells with chromatin breaks or exchanges suffer from disturbances in the anaphase to form bridges. The same is true if nondisjunction due to disturbances of the mitotic apparatus takes place. After telophase, a sizable proportion of the displaced chromatin is not included in the nuclei of the daughter cells, and can be detected in the cytoplasm of these cells by suitable staining techniques. Weber and Legator compared trimethylphosphate in the micronuclei test and the standard metaphase analysis, and found the micronuclei test to be more sensitive and yield response over a greater range of concentrations than the standard metaphase analysis (Weber and Legator, in press). At the present time the micronuclei test seems to be better suited for the detection of chromosome abnormalities than the anaphase procedure.

The Dominant Lethal Test - A dominant lethal mutation is defined as a dominant genetic change that is incompatible with the survival of the conceptus. In this procedure, male laboratory animals are dosed orally or systemically. The treated animals are then mated sequentially with groups of untreated females over a successive period of eight weeks. Females are scored for corpora lutea and implants comprising early deaths and living fetuses respectively.

Heritable Translocation Test - In this procedure reciprocal translocations between nonhomologous chromosomes are observed in primary spermatocytes during diakinesis of the first meiotic division. The procedure can be used to detect reciprocal translocations that have been induced in spermatogonia if the meiotic chromosomes of treated animals are analyzed. The heritability of such translocations can be shown when F₁ and F₂ male progeny of treated males are analyzed. Depending on the breeding pattern it is possible to determine the sensitivity of pre- and postmeiotic stages of spermatogenesis. The detection of translocations in spermatogonia and their mutagenic effect on the progeny is extremely important as the immature cells constitute a permanent population of cells whose alteration is far more significant for the resultant progeny than abnormalities found in later stages of spermatogenesis. Sterility and heritable semi-sterility are estimated in the progeny

and correlated to the translocations. The value of this procedure will only be known after a sufficient number of chemicals are evaluated to determine the frequency of induced translocations so as to define the sensitivity of the procedure.

Repair Studies Carried out in Both Germinal and Somatic Cells - The final expression of mutation lesion is an altered phenotype, and is dependent on a number of factors including the type and extent of the mutation lesion and the ability or inability of the biological systems to repair or alter the modified DNA segment. Initial lesions in DNA, induced by either physical agents or chemicals, can either lead to permanent changes such as mutations or be removed by cellular repair processes. The significance of cellular repair processes is evident from studies of human genetic diseases that are characterized by inability to repair DNA damage. The induction of repair by specific agents could well be one of the most sensitive and earliest indicators of chemicals that have the ability to induce genetic lesions.

The term repair replication is used to describe the insertion of nucleotides into parental DNA strands and this process can occur at phases of the cell life cycle other than at the S phase. Methods for evaluating repair induction can be based on the demonstration of DNA replication occurring at periods other than the normal S phase. The rapidity of the repair process and the extent and the number of bases removed in areas adjacent to the damaged bases can also be used to classify the agent that induces repair.

Specific Locus Test - The specific locus test (Russell, 1951) consists of mating treated and untreated wild type mice to a strain of mice homozygous for known recessive genes. The recessive genes are readily expressed as visible phenotypes in the homozygous state. If a mutation has occurred in any of the test loci in the germ cells of the treated animals it will be detected in the offspring. The value of this procedure in chemical mutagenesis is yet to be determined. The number of animals that must be employed in this procedure and the results with the few agents tested to date does not allow one to make any firm conclusion on the utility of this method. Although there is a high probability that the specific locus mutations are point mutations, the qualitative similarity of the response of the specific locus mutations to that of translocations and chromosome exchanges in response to radiation suggests the possibility that the induced mutations may be the result of deletions arising from two adjacent breaks, rather than one event.

PRIORITY FOR TESTING COMPOUNDS

At first glance the task of screening environmental agents for mutagenicity seems overwhelming. No data is available for the great majority of the hundreds of compounds introduced into our environment over the last 31 years. The appreciation of this formidable task has led to a search for a simple, economic screen to detect mutagens. As stated heretofore it is not logical to utilize simple systems, such as in vitro procedures, to detect potentially active compounds and in fact the most meaningful screen would have to rely on a battery of tests carried out predominantly in intact animals. Since we should not carry out our initial testing to simple economic screens, we must establish priorities for testing environmental agents, and after establishing these priorities proceed to screen the selected compounds in a meaningful manner. A comprehensive screen using a combination of available methods in animals including testing of metabolites produced in the intact host, is still comparatively short and economical when compared to the conventional carcinogenicity screens.

A selection of compounds for testing should be based on the following criteria:

- (a) Exposure of large segment of our population, especially of childbearing age
- (b) The length of exposure
- (c) Persistence
- (d) Structure-activity relationship

The assigning of priorities to environmental agents and their subsequent in-depth screening and characterization offers the possibility of eliminating the most important deleterious environmental agents. In addition to the task of limiting exposure to genetically active environmental agents, it is equally, if indeed not more important, to evaluate all compounds for mutagenic activity prior to their introduction into commerce.

COMBINED TESTING PROGRAM (CTP) IN RODENTS

Combined Testing Program (CTP) is a novel procedure for in-depth mutagenicity testing. In the preceding section a number of the procedures used for detecting mutagenic agents were described. It is now possible to combine many of these procedures in a single experiment without sacrificing the

efficiency or utility of the individual procedures. This approach has become feasible with the realization that the human population is exposed to most environmental agents on a chronic or sub-acute basis. In order to successfully combine many of the known tests for mutagenicity, a single exposure to the chemical can not be employed and the compound is administered in several divided doses over a period of time, typically five administrations of the test compounds are given over a five day period. The multiple administration of the compound over a period of time removes the need for specific timing in any of the procedures employed. In a typical experiment the following studies can be carried out: (a) repair determination in germinal cells, (b) repair determination in bone-marrow cells, (c) repair determination in lymphocytes (d) metaphase analysis of lymphocytes, (e) micronuclei test, (f) metaphases analysis of germinal cells, (g) analysis of blood for active metabolites, (h) analysis of urine for active metabolites, and (i) the host-mediated assay. Additionally an extra set of animals can be included in the test group and used for subsequent mating to determine dominant lethality. The data generated from such a comprehensive screen allows the direct correlation of all the various tests in the same animal under the same set of conditions. In a single experiment we have a comprehensive, detailed analysis of the potential mutagenic activity of the compound under investigation. In a typical CTP experiment only 10% of the animals are used than what would be needed if each of these procedures were carried on individually. Since it is not feasible to rely on any single screen for mutagenic activity this CTP approach may be the most practical, meaningful, and efficient approach to screening chemical mutagens. Where available, Drosophila procedures should be employed in conjunction with the test carried out to detect active metabolites in mammalian blood and urine. In view of recent evidence (Lee et al., 1970; Lee et al., 1972; Browning, 1972) suggesting the feasibility of using Drosophila as an indicator organism, definitive information can rapidly become available on specific classes of genetic alterations induced in germ line cells of a higher eukaryote animal by mutagenic substances found in mammalian body fluids, information unobtainable or obtained only with considerable difficulty from direct tests with mammals, and permit more meaningful inferences to be drawn concerning the kinds of genetic damage likely to be induced in mammalian germ line cells by these substances.

POPULATION MONITORING

In population monitoring, we can define two specific problems: (a) detecting overall changes in gene frequency and (b) determining the contribution of a specific agent or agents in altering the mutation rate.

Our ability to determine changes in the mutation frequency, much less ascertain a specific cause and effect for any single mutagenic chemical in the human population, is extremely limited. The long-term nature of the process, the number of factors that can influence mutation rate and natural selection, and any increase in genetic effects probably would be statistical rather than unique, are among the factors that tend to militate against our ability to monitor effectively the human population for mutagenic effects.

The major approaches to population monitoring are (a) biochemical, (b) cytogenetic and (c) phenotypic. In the biochemical approach a genetically determined variant of a protein (variant allele) is sought. The most promising approach to detecting protein variants is by electrophoretic techniques where it may be possible to screen up to 9×10^4 loci per day (Shaw, 1972). The cytogenetic approach especially when automated, offers the possibility of screening large segments of our population for chromosome abnormalities including the newborn. Once baseline frequencies are established, monitoring for mutations could be initiated. The phenotypic approach is contingent upon the detection of autosomal phenotypes whose occurrence is due to mutations. The rarity of any single phenotypic sentinel, i. e. achondroplasia, Alpert's syndrome, and anurida, and the chances of faulty diagnosis limits the potential value of this approach. The biochemical and cytogenetic approaches therefore offer the greatest possibility for population monitoring. It is, however, doubtful that any of the available approaches will be productive in indicating a specific agent as a mutagen. The one area where meaningful human monitoring is entirely feasible would be in studies with particular subpopulations including high risk groups such as can be found in various chemical industries.

A division of one of the largest chemical companies in this country has embarked on an exemplary program that may well be a prototype for industry to follow. * The cytogenetic approach is used as a basis for identifying chemicals that may produce adverse genetic effects. New employees are cytogenetically analyzed when they are initially employed by the company, as well as at specified periods during the term of employment. Factors that may influence the cytogenetic findings including medical exposure to drugs, radiation exposure, infectious exposure, family history, patients reproduction, etc., are determined prior to each cytogenetic work-up. A specific program has been developed to assess and correlate all of the data generated. During the period of employment, exposure to a chromosome breaking agent can be ascertained and the necessary steps can be taken to eliminate the potential mutagen or to adequately protect the employee from exposure to the toxic agent. Additionally, a number of ancillary benefits have occurred as a result of this program. In the area of cytogenetics we need large field studies

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to determine the base line information. A systematic collection of employees from large industrial concerns will help to establish a large data base. The collection of this type of information also serves to identify, in a unique manner, individuals who can profit from genetic counseling. The cost of this entire program is only a fraction of the cost of an overall industrial hygiene program. This special application of population monitoring will identify a certain percentage of specific mutagens, possible valuable base line data in the field of cytogenetics will be collected, and direct medical help can be offered to specific individuals included in this program.

CONCLUSION

Within the last six years the new area of genetic toxicology has come into being. The emergence of this area occurred as procedures were devised for detecting mutagenic activity predominantly in animals or in animal derived material. Additionally, the development of the animal testing procedures was found to correlate, to a high degree, with results obtained from Drosophila. The optimum protocol for genetic toxicology would include a battery of tests carried out in animals or where the in vivo metabolic products of the animals are tested. Drosophila is a meaningful addition to the testing protocol. It is not wise at the present time to rely on any single procedure to indicate chemically induced genetic damage. Our ability to accurately determine genetic damage can only be assessed after quantitative data from a number of the best available systems are analyzed. Just as animal tests are essential for detecting mutagenic activity, mammalian cell cultures and various sub-mammalian procedures are of value in characterizing the induced genetic lesion. The ability to characterize the induced change is a unique attribute of this area of toxicology.

The significance of the hazards posed by chemical mutagens to the welfare of our populations can not be determined with any degree of accuracy. It has been estimated that 15 million Americans suffer the consequences of birth defects of varying severity and that of this number 80% and therefore 12 million Americans carry genetic diseases wholly or partly due to alterations in gene or chromosomes. In a specific category of genetic syndromes, autosomal dominants, we can by pedigree analyze what percentage of the total number is due to newly arisen mutations within this generation. Approximately one-fifth of the analyzed dominant diseases are newly arisen mutations. It is not possible however by presently available techniques to determine what fraction of the newly arisen mutations, for this or any other category, is due to chemicals as opposed to radiation, virus, or spontaneous mutations. It is safe to suggest that environmental agents play a major role as a causative factor in our

overall genetic disease burden. With a minimal number of compounds tested in animals we have already identified several which can induce mutations. In succeeding years we will find that animal testing will serve as our first line of defense for detecting environmental mutagens, and, in all likelihood we will identify potential chemical mutagens in high risk exposure groups such as employees of certain industries. The high correlation between carcinogenic and mutagenic compounds (Miller and Miller, 1970) means that decisions concerning the elimination of genetically hazardous chemicals from our environment can be reached on the basis of results from currently employed procedures, and that the validity of these decisions can be strengthened by the development of new procedures in the area of genetic toxicology.

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OPEN FORUM

DR. CROCKER (University of California, Irvine): I have a question to ask Dr. Weisburger. When one is setting out to do a carcinogenesis test, does one determine an LD₅₀ first? If so, how does one use the LD₅₀ information to arrive at a dose schedule for carcinogenesis testing?

DR. WEISBURGER (National Cancer Institute): Well, maybe I should give that question to Dr. Magee because he has written a beautiful chapter in a book about chronic toxicity. We do use the LD₅₀ data to some extent but that's only to show us what dose level we cannot go above in setting the chronic test doses. The final doses are based on what one observes over a 30- to 90-day period following animal weights and their general appearance and also doing a histopathological study after the 90-day period to see which organs were affected. Then we use the dose in which there was no demonstrated effect as the top dose. In fact, we try to retreat a little from that dose to insure survival.

DR. CROCKER: This is after a 90-day study?

DR. WEISBURGER: Yes.

DR. CROCKER: So first there may be an acute LD₅₀, then a 90-day study, and then selection of the top ineffective dose for essentially a lifetime or a two-year study.

DR. WEISBURGER: We're now testing some compounds which we know are persistent. We're asking some of our contractors to determine body residence times, to give us a rough idea whether there is a buildup of material which might lead to toxicity problems at a later time.

DR. SMITH (University of Cincinnati): Are compounds for testing always given in the diet?

DR. WEISBURGER: No, compounds are given by gavage or in the drinking water also, if it is indicated. Compounds which are liquids or are especially unstable are given by gavage. We don't always mix the materials in diets.

DR. SMITH: I was wondering because many compounds have a very rapid turnover and, therefore, once a day dosing can be a real mistake. I mean you really are not treating the animals very much of the time. Methadone wasn't thought to be a compound that produced tolerance in rats because they were treated once a day, five days a week. The first papers by the researchers at Lilly said it wasn't addicting or didn't induce tolerance simply because the method of application was inadequate.

DR. WEISBURGER: There are quite a few instances where the effect is different if the compound is given in the diet or by acute ingestion but when you have an unstable compound or a liquid, which is the better course to take? One always has to weigh all the possibilities.

MR. WANDS (National Academy of Sciences): I'd like to ask Dr. Weisburger if she would comment briefly on her personnel protection procedures for her laboratory workers handling all of the known and potential carcinogens.

DR. WEISBURGER: The diets, if diets are used, are mixed in machines which are kept in hoods which have filters so that the air is drawn through the filter to the outside during the mixing procedure. The people who handle the animals and feed the animals wear coveralls and boots, shoe covers. They wear hats and face masks and gloves and they are supposed to shower after they leave. They are not allowed to smoke or chew tobacco or anything of that sort while taking care of the animals or handling these diets which contain compounds that might be potential carcinogens. Those are some of the protective measures that are taken. Most of these workers are employees of contract laboratories where our bioassay programs are conducted.

MR. WANDS: How do those requirements compare with the OSHA standards for handling their 14 carcinogens? Are they more stringent than the OSHA regulations or less so?

DR. WEISBURGER: Well, some of our contractors are fortunate enough to have facilities which I think measure very favorably to the OSHA standards. For example, we have a large program at Fort Dietrick and they have an extensive program there of showering in, changing clothes completely and then being present only in either the clean or the dirty corridors so that there is no mixing. They are completely protected and then they shower out again. Quite a few other contractors have comparable facilities so I think our contract laboratories measure quite favorably with the OSHA standards.

DR. GROTH (National Institute for Occupational Safety and Health): When you consider what route by which you are going to give the carcinogen, do you consider the route by which man is exposed, particularly in industry?

DR. WEISBURGER: We do take this into consideration. Compounds which would be food additives would preferably be given in diet. But, of course, you have to realize in many cases, as long as the compound gets into the organism, very often the effects are going to be very similar. I can think of examples with hamsters and many of the nitrosamines. No matter how the compound was given, they always affected one specific site.

DR. HENDERSON (Olin Corporation): I'd like to follow up on this just one more step. Some of the materials do get excreted. Do you check the excretable levels and how are the excreta handled if they do have a carcinogen?

DR. WEISBURGER: In the large scale bioassay programs, we haven't done any excretion studies. Usually the animal wastes are put into large impermeable bags and incinerated.

DR. CANTOR (Environmental Protection Agency): I wonder if you could throw some perspective on the animal screening tests with respect to other kinds of carcinogen screening tests as sponsored by NCI. If I remember your figures right, you mentioned that you're conducting tests on about 400 compounds at a total cost, after it's all counted, of about \$100,000 each, which amounts to something in the range of \$40 million for these 400 compounds. I wonder, if in comparison to that, how much NCI is spending on development of alternative screening systems and to what extent they're now being used in a regular fashion or might in the future be used.

DR. WEISBURGER: I would like to point out that we are not starting 400 new compounds each year, some of them are ending and we are phasing them out. As those go out, we try to phase in new compounds so that we keep the level more or less constant. The cost of that \$80,000 to \$100,000 is for each compound over the 3-year period that it requires. As for the relative amount of money that is spent on developing new assays, I would say that we've spent about \$250,000 perhaps on developing some mutagenicity tests and tests of that type. I don't recall exactly how much money is being spent for the development of some of the cell culture assays and combinations of in vivo and in vitro tests, such as DePaulo is doing. The mutagenicity studies are the only figures that I can approximate.

DR. CROCKER: Dr. Magee, you might care to discuss that point since you are also doing some direct carcinogenesis testing. Are you developing any parallel "quickie" methods?

DR. MAGEE (Middlesex Hospital Medical School): Quite a number of methods of these kinds are being looked at in the United Kingdom. I think that, like you, we would feel that at the present time, there is no method that has the certainty of the conventional long-term feeding study.

MR. VERNOT (University of California, Irvine): Dr. Weisburger, as I recall, the greatest numbers of animals you used in your studies were either rats or mice. Do you feel that there is any danger at all that you might miss a chemical that might be carcinogenic to man by limiting yourself to just 2 species?

DR. WEISBURGER: I don't know of any chemicals which have turned up lately that didn't cause tumors in either one or the other. Of course, we have considered using hamsters for further studies, but we just haven't had the money to develop those in greater detail. In view of Saffioti's work with the hamster and aromatic amines, we thought maybe the hamster could substitute for the dog in many such studies. We have not yet started such a program.

DR. RANADIVE (U. S. Army Environmental Hygiene Agency): This is along the same line as Mr. Wand's question for you, Dr. Weisburger. For employees exposed to carcinogens, the OSHA standards also require appropriate medical surveillance, whatever that is. How do you medically follow the workers and what is your experience?

DR. WEISBURGER: The Department of Health, Education and Welfare is now developing safety standards for the handling of chemical carcinogens in our own laboratories. The standards include medical surveillance requirements with a physical examination before the employee starts a new project. I do know of one contractor who was involved in preparing reference standards of various chemical carcinogens that certainly did have a very thorough physical examination for their employees which included just about everything. These people, because they are using known chemical carcinogens, have a repeat physical examination every six months as a routine follow up. The HEW standards will include preemployment physical exams and a follow up at least once a year.

DR. GROTH: In view of the importance of diet on the effect of a chemical and its carcinogenicity, do all the contractors use the same diet? If they do, how was the composition of this diet selected?

DR. WEISBURGER: At present, most of our contractors in the bioassay program use the Wayne Meal which is manufactured by Allied Mills in Chicago. This doesn't mean that we think this is the very best diet but it seems to be one which is more consistent in composition than some of the other animal diets which are available.

DR. MAGEE: I just wanted to ask Dr. Weisburger, are the diets in general monitored for aflatoxins and nitrosamines?

DR. WEISBURGER: One of the contractors in the bioassay system is an analytical laboratory which does nothing but analyze the compounds selected for bioassay or develop methods, check stability on storage and in animal diets. They do check the samples of the diet from the contractors who are doing the actual bioassay for compounds like pesticides and the mycotoxins.

MR. WANDS: That was exactly the point I wanted to make - that many of us in this audience heard the papers at the Gordon Conference this year in which there were several reports of rather frighteningly high levels of pesticides and other similar materials in the standard laboratory chows of most reputable dealers. These varied extensively from day to day and batch to batch. This could, of course, affect your data tremendously. I am glad you are monitoring that very carefully. It is quite critical.

DR. BACK (Aerospace Medical Research Laboratory): What about estrogenic substances in your feeds? It seems to me that many dog and rat foods have been high in estrogenic activity. As a matter of fact, a few years ago in Oklahoma, we did some studies looking at digitalis effects. We were getting tremendous amounts of estrogen from our feed. Since sex differences are found, are you monitoring for these things?

DR. WEISBURGER: We don't specifically look for estrogens, although we've asked our analytical facility to look at one of these compounds in moldy corn, zearalenone, and see whether they can develop an assay for that.

DR. JACOBSON (National Institute for Occupational Safety and Health): I'd like to suggest some attention be given to added antioxidants also since they can inhibit the formation of cancer.

DR. COURI (Ohio State University): With all of the comments about diet, I feel I am compelled to say something about it. Wouldn't it be wise, since all of these experiments have control animals on the same diet without the suspected carcinogen, to not worry too much about the emphasis of a diet which is entirely nutritional and clean if the model is for humans? What is our diet like?

DR. WEISBURGER: I think that the human diet depends a great deal upon individual preference. You may like pizza and charcoal broiled steaks, but I'm not a lover of steaks.

DR. GROTH: I became interested in this problem for the same reason that Dr. Couri stated. The rat diet is quite a bit different from the human diet, for reasons which sometimes are rather vague. In commercial diets, the inorganic compounds which are added to help the rat to reproduce and to gain weight rapidly are selected because they are cheap. Consequently, the diets contain 150 to 400 ppm iron, about 70 to 120 ppm manganese, 10,000 or more ppm calcium and several other ingredients, including about 0.5 ppm selenium; whereas the normal human diet contains a tenth of a ppm selenium, about 3 ppm manganese, about 25 ppm iron, and about 3.5 ppm copper. In contrast, the rat diet has about 10 ppm copper. I could go on with these but that's enough as an example. Now we don't know whether or not the iron, manganese and other things will affect carcinogenesis experiments, particularly by dietary administration. We do know, however, that injecting manganese metal with nickel sulfide will inhibit

rhabdomyosarcomas. We know that copper does have some effect on carcinogens. We know that rats on a magnesium deficient diet develop lymphosarcomas. This might be relative, the amounts necessary to inhibit it might be relative to the dose of the carcinogen.

DR. HOMAN (Environmental Protection Agency): In regard to Dr. Back's point about estrogens in the feed, I happened to pick up a copy of Chemical and Engineering News during the break. There's an article in it which discusses the fungus responsible for mold of corn, which in addition to producing a toxin also produces what they refer to as copious quantities of an estrogenic material which can cause estrous and an anabolic response.

DR. CROCKER: That estrogen may be what Dr. Weisburger was referring to.

DR. WEISBURGER: Yes, it's a multi carbon ring compound with a few oxygen atoms on the sides, and it does have an estrogen-like effect.

DR. MAGEE: Dr. Weisburger, has zearalenone been tested for carcinogenic action?

DR. WEISBURGER: No, it has not been tested for carcinogenic activity, although Dr. Shanetell has recommended that it be done.

DR. CROCKER: In the midst of what appears to be a great deal of interest but rather small numbers of answers for all the questions that we have, what would you recommend as a procedure that we might follow to encourage uniformity, at least within the research establishments that are doing this in this country?

DR. WEISBURGER: Well, I don't think we can regulate uniformity but one has to take a reasonably good diet and try to hope for a reasonably consistent composition. I might add that apparently the NCI is going to spend quite a bit of money in looking into the problems of diet and carcinogenesis within the next year or so, and at least hold a conference on it to see whether further work should be done in this area and try to collect some of the possible answers.

DR. CROCKER: Would it be appropriate while some of these decisions are pending if most of us were to try to follow whatever method the NIC is using now, and if so, is that method including some of the details of the diet and the like, published and available for general use?

DR. WEISBURGER: Copies of our protocol can be obtained from Dr. Page who is the director of the Carcinogen Bioassay and Program Research Branch of the National Cancer Institute.

MR. WANDS: I would raise the question which is perhaps a precedent of the one you asked. Should there be a standardized protocol for carcinogenic testing or is it better to allow the individual scientists a certain degree of freedom to pick and choose provided he has suitable controls, positive and negative in his experimental program?

DR. CROCKER: I think that the guidance that Mr. Wands mentioned was the general position of not seeking to impose uniformity of practice but to at least assure positive and negative controls. It may be an answer. The additional reason why I think that may be very important is that Dr. Newberne, in his presentation, included one table showing the variation by year of spontaneous tumors in mice. This was over a period of several years of observation. It's obvious that in some laboratories where a control group of animals may be backed up by looking at many animals over their lifetimes, not necessarily in parallel with a single experiment but in parallel with a number of experiments, may arrive at a general experience of control data that may not be relevant to the particular exposure underway. This has its merits and its possible demerits. Would anyone care to comment on the appropriate method of developing a control set of data in the face of the variables that were shown in Dr. Newberne's presentation? What would your approach be to a standardized control population? I see someone willing to answer that question.

DR. BINGHAM (University of Cincinnati): I would like to comment since Dr. Weisburger did not pick this up. You could call it a defense of the National Cancer Institute. They have a standardized procedure that they have many of their contractors hold to because they want comparable results, but I assure you that they give many contractors a lot of latitude, particularly to Universities in the design of their own experiments. Some investigators are allowed to put in some variables, such as varying the diet, using their own judgment, as long as they have adequate controls and what they plan to do does seem reasonable. It is not just a cut and dried bioassay program. They have many other aspects to the program. Dr. Weisburger might want to verify what I'm saying.

DR. WEISBURGER: That's entirely true. In fact, Dr. Newberne's project is supported in part by the National Cancer Institute.

DR. SMITH: One way to avoid so many variables would be something I've been trying to find somebody interested in and that is to actually provide a uniform defined diet. Now, nobody could use this for every purpose nor would I even want to recommend it. But it would be nice to know that you have a fairly stable diet for doing special tests. I wondered if the National Cancer Institute has ever addressed itself to the possible formulation for a really standardized diet and then couple that with a standardized animal which really is fairly available. This is not perfect by any means,

but still I'm sure that the statistician trying to handle the data would be a lot happier if we could reduce the variables rather than just allow us to play with them in any way we feel.

DR. WEISBURGER: Dr. Newberne should really be here because he's the leading proponent of a standardized semisynthetic diet which would start with a pure casein and dextrose and ingredients of that sort where one can modify or analyze the materials and have a defined diet. But the expense of that sort of diet for all the animals that we use would be so great that we couldn't support the bioassay program. Now that sort of diet is fine for a basic research program, but if one's looking at 400 compounds to see whether they are or are not carcinogens, the cost might be astronomical and we just couldn't afford to do that sort of thing.

DR. CROCKER: I wonder if we might move now from the questions on diet and bioassay, which we could continue to discuss for a long time, on to the subjects that might have been raised by Drs. Magee, Shank and MacEwen in their presentation of data on the hydrazines and nitrosamines. I had one question that I would like to ask of Drs. Shank and Magee. Do you believe the data presented by Dr. MacEwen were adequate to establish carcinogenicity for hydrazine, and if so, why is hydrazine carcinogenic?

DR. MAGEE: I think that the first question is very difficult to answer because it is very difficult to know what carcinogenesis means, certainly that there was an increased incidence of tumors. But why is hydrazine carcinogenic? Well, of course, again I have no idea. I don't think any of us really has any idea how any of the carcinogens work. An idea that has been put forward, not originally by me but by others, it's conceivable that hydrazine itself might be methylated in the body. Quite a few compounds are methylated in the body. I don't support this, partly because of some studies that we have done of the acute effects of monomethylhydrazine and 1,2-dimethylhydrazine on the liver. We find that the ultrastructural effects of 1,2-dimethylhydrazine are very similar to dimethylnitrosamine, whereas monomethylhydrazine is quite different and it is more related to what one ascribes to the effects of hydrazine itself. So I think that they do work by different mechanisms but I really cannot suggest at all how hydrazine causes tumors apart from the suggestion that I have made. Perhaps Dr. Shank can make some more suggestions.

DR. SHANK (University of California, Irvine): Dr. Magee and I were discussing this matter with some other people and I recalled a very preliminary experiment that Dr. Viatraveno did in Professor Magee's laboratory. I'm not exactly sure of the details of the experiment but Dr. Viatraveno was very interested in alkylation of nucleic acids and he was trying to find a compound that would cause necrosis of the liver but couldn't possibly alkylate DNA as a control toxin. He chose hydrazine and found that administering hydrazine to rats did increase the amount of 7-methyl-guanine of RNA and I think DNA as well. He repeated the experiment again with hydrazine, this time using C₁₄

labelled methionine. And the 7-methyl-guanine that resulted in DNA was radioactive. The first thought was that perhaps hydrazine was methylated so then he tried phosphorus as a compound that caused necrosis of the liver but couldn't possibly be methylated. The question was: if he gave phosphorus, would phosphorus produce 7-methyl-guanine and liver DNA? The result was yes, it does. He gave phosphorus and radioactive methionine and got radioactive 7-methyl-guanine in the DNA. We have repeated this in our own laboratory with the phosphorus and methionine and have seen the same thing. We gave them unlabelled aflatoxin and radioactive methionine and found 7-methyl-guanine radioactive in DNA. We've done this with dimethylnitrosamine and radioactive methionine, again getting radioactive 7-methyl-guanine in DNA. I don't know how to interpret this. One suggestion is that there may be an induction of a DNA methylate by the toxic insult itself, and that the toxin does not have to form an alkylating agent itself. All it has to do is induce methylation. In that case, hydrazine could be justified as a carcinogen and not destroy the scheme that I presented.

DR. CROCKER: The reason that I was interested in the scheme was that it was a basis upon which to rationalize a single path of final action for symmetrical dimethylhydrazine, monomethylhydrazine, and I believe, unsymmetrical dimethylhydrazine. My reason for continuing this discussion, therefore, is to determine whether we can project from the rationalization by which several compounds were found to act similarly, a relatively lesser risk of carcinogenicity in those compounds of near molecular similarity. Now, obviously, testing for hydrazine has demonstrated that it is so that the failure of that rationale does not necessarily eliminate hydrazine as a potential carcinogen on any theoretical grounds. We are now left with unsymmetrical dimethylhydrazine as an interesting question, which again is only going to be answered by direct testing. Do I understand you correctly when I say that because you worked out one positive rationale, you have not thereby excluded any of the other compounds which might not fit it?

DR. MAGEE: You may have misunderstood one thing. We do not suggest that MMH acts in the same way as symmetrical dimethylhydrazine. What we demonstrated was that while it may act as a very weak methylating agent, its potency is not to be compared with 1,2-dimethylhydrazine. The conclusion we came to was that 1,2-dimethylhydrazine seems to be metabolized in a different way from the other hydrazines, including hydrazine itself, and 1,2-dimethylhydrazine does seem to work, in our view, at a molecular level in the same way as the nitrosamines do. Of course, one must accept that there are many ways in which a chemical can induce cancer. They don't all have to work like a nitrosamine, not even to me. Since Dr. MacEwen has produced further evidence for the carcinogenicity of hydrazine, I would say this is acting in a different way. But, equally, I would say that that sort of evidence of pulmonary adenomas and hepatomas in mice may perhaps be regarded as a weaker carcinogen than something like a nitrosamine. How can one really extrapolate this? I don't know, because it may be that weak

carcinogens are even more harmful to man than powerful ones because they are insidious and less easy to detect perhaps.

DR. CROCKER: Well, I think what is left open then is the possible carcinogenicity of UDMH on which the evidence is, at this moment, nothing but pulmonary adenomas in the lungs of mice. Further testing is in progress as it is with hydrazine. It does raise an interesting question which I think Dr. Winstead or Dr. Thomas might speak to. In the use of either hydrazine or UDMH as a fuel, what is the likelihood that any residues of these would remain after ignition?

DR. THOMAS (Aerospace Medical Research Laboratory): You're concerned about any possible unburned fuel in the exhaust products of a ballistic missile during firing. Is that correct? If we wouldn't have 100% combustion in a rocket engine, we wouldn't be flying them very successfully. The whole purpose of ballistic missilery is based upon thermodynamic calculations that you are going to hit a target because it's going to fly where you figured it would on the basis of total fuel and oxidizer load in that missile. Yes, there is a guidance system but the range would not be accurate if you would not have 100% combustion and, therefore, I don't expect any unburned fuel in an ordinary firing of a ballistic missile or a test firing. In the presence of a very strong oxidizer like N_2O_4 , and a system in which you would have anything but complete combustion, then you are down to CO_2 , nitrogen, hydrogen, oxygen, and so on in the exhaust, nothing complicated which would look like a carcinogen.

DR. MAGEE: I wonder if Dr. Thomas could tell us, can he distinguish between 100% combustion and shall we say, 99.5% combustion? This is quite a naive question, I know nothing about this.

DR. THOMAS: No, I'm afraid I'm not a rocket engineer, but I think that if you're talking about, let's say a 4000-5000 mile range, a 99.5% combustion rate could make a difference of several miles in distance at the point of impact and that would be unpredictable. It would depend on a lot of circumstances which you could not tolerate in system design, but that's how far I'm going to stick my neck out. I'm not intimately familiar with thermodynamic calculations and ballistic missilery.

DR. SHANK: Does anyone here know what the fuel was for the moon rockets? I thought the comment Dr. Magee made this morning, that there was dimethylnitrosamine found on moon rock specimens was very intriguing. The only way I could explain DMN getting on a moon rock would seem to come from oxidized rocket fuel from the moon. Does anyone know? Was 1,1-dimethylnitrosamine a component of the fuel?

DR. CARTER (Aerospace Medical Research Laboratory): The pulsing engines on the Apollo module used monomethylhydrazine as the propellant.

The Lunar module, as I remember, used Aerozine 50 which is a combination of UDMH and hydrazine. They also have attitude engines. These are pulsing engines and I'm not sure but I think they used MMH. This is just to change the attitude of the module itself.

DR. THOMAS: Is it a monopropellant system?

DR. CARTER: I'm not sure. I wouldn't want to answer that.

DR. THOMAS: But the presumption is that there is an oxidizer present as well?

DR. CARTER: I would think so. I don't know how otherwise it would work.

MR. VERNOT: That presumption isn't necessarily correct, because the hydrazines are thermodynamically unstable at room temperatures. All they need is a catalytic surface and they'll decompose to nitrogen and methane and other gases. This is the monopropellant use of these particular materials.

DR. CROCKER: In any event, if there is no other fuel component for purposes of oxidation, there is a catalyst for that purpose.

DR. MAGEE: I think there is something that ought to be mentioned. I didn't mention it this morning in my paper. I think what I said was that MMH had not been shown to produce colonic tumors, which is the main tumor that you get with 1,2-dimethylhydrazine. Dr. Bela Toth at the Eppley Institute in Nebraska has published work showing that MMH does, in fact, produce vascular tumors of the liver. I don't know of anybody else who has found this, but I think this should be kept in mind regardless of mechanism. I mean according to him, and we heard from Mr. Wands this morning, the importance of not disregarding inconvenient results that get published. Dr. Toth would say that MMH is a carcinogen, but it's not the same sort of carcinogen in our view as 1,2-dimethylhydrazine.

MR. WANDS: I'd like to try to get a handle somehow or other on the translation of these various animal data on the carcinogenicity of hydrazine into potential human experience. As I recall, the concerns about carcinogenicity of hydrazines in the very beginning involved the use of isoniazid, an antitubercular drug, which had been through quite a few clinical trials. Animal toxicity tests were conducted at the same time and isoniazid did produce some tumors in the animals. These tumors were then later identified as being due to in vivo hydrolysis of the isoniazid to release hydrazine. This then led to an investigation of hydrazine and other derivatives of it including the propellants and many others. One of the things that was tested was hydrazine sulfate which also came up positive by some of the tests. I'm wondering if there have been any epidemiologic

studies conducted on hydrazine or isoniazid. I don't know whether anyone here in the room would have the evidence, perhaps Dr. Weisburger would. Have there been any epidemiology studies on these TB patients who have been treated with isoniazid for quite some time and is there any increased tumor incidence of any kind in these people?

DR. WEISBURGER: If I remember correctly, Shirley Farrenby did such a study and found that there was no increase in tumor incidence. You may wish to contact her. I'd like to add that we tested hydrazine sulfate in newborn rats and newborn hamsters. I also think that Peacock in London tested it in newborn hamsters and found no tumorigenic effects.

DR. SMITH: I was interested in the work that was presented by Dr. Shank. How do you account for the localization of the effects of these different compounds, specifically in the esophagus? I would like for you to mention, if you would, something more about the comments you made about the fact that it was like the MFO (mixed function oxidation), but that it was not MFO.

DR. SHANK: We can't. As least I can't explain all of the target specificity of the nitroso compounds. For the most part the tissue's ability to metabolize, at least oxidize the compounds, seems to correlate very well with the response of carcinogenesis. This theory is supported by the action of the nitrosamides which break down spontaneously and tend to produce local tumors much more so than the nitrosamines which require metabolic activation. I don't know that there is a 100% correlation between metabolism and tissue specificity but for most cases in which this has been examined, it does hold up fairly well. Perhaps Professor Magee would like to make further comment on that. On the second question on why I implied that perhaps the enzyme system that metabolizes dimethylnitrosamine is not exactly the same as mixed function oxidases that metabolize a great many drugs, comes from observations that phenobarbital pretreatment induces the mixed function oxidase system. If you pretreat animals with phenobarbital, and then give them hexobarbital, they will hydroxylate these drugs at a rate which you can check kinetically and it holds up very nicely. Then if you do the same thing, but the drug you administer is DMN, you would expect greater oxidation of DMN and in fact, you don't see this. You get a decreased production of formaldehyde from DMN, the opposite effect of what you would expect to see. This has been confirmed now in several laboratories. This would tend to imply there is some sort of a difference between the two enzyme systems.

DR. HODGE (University of California, San Francisco): May I ask Dr. Magee if he'd comment on the point that he referred to this morning concerning the formation of nitrosamines from nitrites.

DR. MAGEE: This is now a subject that is exciting a great deal of interest in many places. There are at least two ways in which nitrosamines

may occur in the human environment. One is by interaction in foods to which sodium nitrite has been added as a preservative where interaction may occur with various secondary or tertiary amines that are present in food to give rise to nitrosamines. This has been clearly demonstrated. These studies arose out of some observations in Norway where quite a large number of sheep died one winter for an unexplained reason. They had severe liver damage and it turned out that they had been fed diets of fish meal preserved with sodium nitrite. Fish, of course, contains dimethylamine and trimethylamine, particularly slightly decaying fish, and these compounds had reacted with the nitrite as one might expect just from straightforward chemistry. There were toxic amounts of dimethylnitrosamine present in this fish meal. A similar incident happened in the United Kingdom where a number of mink were accidentally killed which led to a very prolonged law suit in the high court. Ultimately, even the judge ruled that there was nitrosamine formation so you can take it as an official act. There are a number of studies that I'm sure you are all familiar with of small amounts of nitrosamine found, particularly in food like bacon. The amount of nitrosamine present increases when the bacon is cooked. Another way in which this can happen is that you may get a reaction in the stomach itself, since the nitrosation reactions are acid catalyzed, when amines are ingested. Nitrite is always present in the stomach because there is quite a large amount of nitrite in the human saliva. Recent work at MIT by Dr. Stephen Tannenbaum, for example, has confirmed this point. So this raises the question of to what extent will all of us having nitrosamines formed in our bodies from inert precursors, such as nitrites, and various secondary and tertiary amines. The study of this subject is still in its infancy and I don't think I can really comment on how significant it might be for human cancer at this moment. But certainly I think it is worthwhile having a lot more studies devoted to it.

LT. ANDERSEN (U. S. Naval Toxicological Unit): I have both a comment and a question. The comment is there is one way that dimethylnitrosamine could have been found in a moon rock. That is that UDMH is made by hydrogenating dimethylnitrosamine and dimethylnitrosamine is left in the UDMH. After burning, the hydrazine would be preferentially oxidized and passing through as an unreacted product might be the nitrosamine. And it could be picked up for quite a prosaic reason. The question I had for Dr. Magee is: if the reaction in the stomach is acid catalyzed to form nitrosamine, what is the reaction in meat when you add nitrite? Do you also suppose that is acid catalyzed or is that a different reaction?

DR. MAGEE: I don't know a lot about this but I don't think it is a different reaction. I simply believe that it is the same reaction taking place much more slowly but there is a lot of time for it to happen.

LT. ANDERSEN: Do you believe that the reactions can occur in situ in any other place except the stomach? Do you believe it occurs, for instance, in the liver itself?

DR. MAGEE: I don't know about the liver but there is experimental evidence that it can occur in the infected bladder. This is because nitrosation can be catalyzed by certain microorganisms. There are several papers on this subject and some from Professor Williams' laboratory in England. Michael Hill has shown, quite convincingly, that dimethylnitrosamine can be formed in urine of infected rat bladders.

DR. WEISBURGER: Professor Klubes at George Washington University found that there were certain bacteria in the intestinal tract which could carry out this nitrosation. Dr. Kieffer at NCI is also studying the nitrosation and finds that some neutral substances such as formaldehyde can catalyze nitrosation. There may be many other factors which make this reaction go that we aren't aware of.

DR. HENDERSON (Olin Corporation): I think I would be remiss since I am employed by one of the major hydrazine producers in the U.S. not to ask what the plans of the military are to change their human monitoring of those people handling hydrazine. What do they plan to institute as new procedures on their periodic preplacement physical examinations for those workers?

DR. CROCKER: I'm not sure there's a spokesman here who could field that question but I think that I would be willing to say that it's been deeply considered and if there's no one here who knows what the outcome of those considerations are, it will continue to be.

DR. STOKINGER (National Institute for Occupational Safety and Health): I have a very simple question for Dr. MacEwen. I listened pretty closely to what he said but I don't think he compared his present reported experiment with work that you did, Dr. Back, the 90-day continuous exposure of animals to hydrazine at its TLV concentration. Did you get identical results in this study with that done some years ago?

DR. MAC EWEN (University of California, Irvine): If I understand correctly, you're asking me if I got identical results in the 1 ppm continuous exposure group. I did not get identical results. In that previous study, 99% of the rodents died within a very short period of time. There were some possible reasons for those deaths in their experiment. Our chambers, as you know, are very well controlled in terms of temperature, humidity and air flow. The chambers in that other study were not that well controlled. They had some heat excursion problems which probably enhanced their mortality rate. The increased deaths were probably due to overheating rather than to hydrazine, or to both factors in combination. As you know, you can enhance the lethality of any compound by mild increases in ambient temperature.

DR. STOKINGER: The next question I have is: do you feel, as a result of this recent study of hydrazine, that is the intermittent exposure of animals at 1 ppm, that you'd give consideration to reducing the threshold limit value?

DR. MAC EWEN: At the 1 ppm intermittent dose, and its comparable continuous dose at 0.2 ppm, we found something like a 35% incidence in tumors which was considerably above our control rate. At the higher levels, 5 times that, we had an approximately 80% tumor incidence rate. Now, you couldn't tell that from the table I had up there. I showed the total number of mice with tumors and numbers exposed. I'm not the man who should answer that question. You are. You are the chairman of the Threshold Limit Committee.

DR. STOKINGER: I looked at your data and I thought one of the controls had a tumor.

DR. MAC EWEN: Yes, one control.

DR. STOKINGER: Two out of eight have the tumors in the exposed group at 1 ppm. I didn't think that was very much of a difference.

DR. MAC EWEN: It was 1 out of 8 versus 3 out of 8. There is a dose response curve here if you look at it. The other thing is you must remember this experiment wasn't initiated to determine the carcinogenicity of this compound but it was to look at the suitability of the TLV. There were other effects observed. There were increased deaths at the TLV concentration in mice and considerably higher mortality in mice at the higher dose level.

DR. CROCKER: I think it's safe to expand Dr. MacEwen's last comment to the effect that the experiments are being extended in larger numbers of animals to clear up this question of the small numbers of controls that were positive as compared to the lowest dose response.

DR. GROTH: I'd like to ask you, in view of the fact that most chemicals are not very pure, how pure must a chemical be in chronic testing so that one is sure that a carcinogen is the major constituent and not the contaminant?

DR. WEISBURGER: I suppose that question is for me. We don't put any limits on the purity of a chemical. In fact, if we can only obtain the technical material, we do use that for the bioassay systems, but we certainly do try to find out by analysis what all the constituents of the mixture are. Then if it does turn out to be carcinogen, we can go back and try to identify which component was responsible.

DR. RANADIVE: Dr. Henderson questioned what the military was doing with respect to people exposed to hydrazines because of Dr. MacEwen's findings. Should one do a cold hard epidemiologic study in the humans that are exposed to hydrazines?

DR. MAGEE: I think the answer is obviously, yes! I do feel that the only way in which we will really come to understand and interpret all of this animal data is through more human epidemiology. I'm quite convinced of this point and I think hydrazine would be a good example if the situation is such that it can be done in a meaningful way.

MR. WANDS: I would urge, not only Dr. Ranadive and the Army, but all of the other military services to take full advantage of their unique populations that they have for carrying out retrospective as well as prospective epidemiology studies on some of these highly unusual chemicals. I know that the services do provide good industrial hygiene practice, particularly for hydrazines because of their well known toxicity to say nothing of carcinogenicity. Nevertheless, they do have an opportunity to follow people for a long, long period of time. The Navy, so far, in their submarine program for example, has not followed these people up as well as might be done. I think that in most of the military munitions plants, both military owned and operated as well as military owned, contractor operated that there have not been, until very recently, any epidemiology studies. Here is a tremendous reservoir of data that is going uncollected and I would certainly urge very strongly to anybody who is listening and anybody who may read these remarks later that we do collect these kinds of data. That is the only way that we are going to be able to identify whether or not we have false positive or false negative results in our animal data and thus contribute to our reliability of our predictions.

DR. HENDERSON: I can speak for the corporate medical department and say that we are trying to look at all of the people we have ever employed in our hydrazine plants. We have, I believe, 7 people who have been in our Lake Charles, Louisiana hydrazine plant since it started operating. I know of 3 people who started working with hydrazine in our research operations at Niagara Falls about 1946. One of these just retired and we made sure that we got good preretirement medical history on this man. I cannot speak for other hydrazine manufacturers officially; however, we are meeting on an international basis to try to see what epidemiologic information can be put together from all of the possible industrial sources of hydrazine exposure.

DR. CULVER: I'd like to ask Mr. Wands to make his guess as to what will be the next industrial carcinogen to take vinyl chloride's place.

MR. WANDS: Are you asking what is going to be the next OSHA carcinogen to be published or the next crisis carcinogen?

DR. CULVER: The next crisis carcinogen.

MR. WANDS: Your crystal ball is at least as good as mine. There are several potential materials which might well come up this way. We've

just been discussing one class of them here this afternoon extensively, the hydrazines and nitrosoureas. Certainly there are other industrial chemicals of which we have indications that they may well be carcinogenic at high enough doses or with long enough exposures. We spoke a little bit this afternoon of one of these, benzene. Now we could perhaps split hairs, if you wish, and say that leukemia is not a carcinogenic response but that is purely hairsplitting, and I wouldn't be at all surprised to see OSHA doing something about this matter. This may well be the trigger for official action by OSHA. We've got roughly a thousand compounds, as I said earlier today, that are known animal carcinogens of varying degrees. Any one of those might be the next one to hit us by surprise. Selikoff has been predicting a deluge of mesotheliomas in the people exposed to asbestos during our massive program of ship construction in World War II when we said, "Damn the torpedoes, damn the asbestos, full speed ahead, and get those ships afloat." We weren't too fussy about our industrial hygiene protection at that time. A lot of this exposed population has been difficult to follow up, but Dr. Selikoff has located some of them. The epidemiology data out of England, where they also went through this phase of ship construction much earlier than the U.S. did, is beginning to show an increase of pulmonary cancer of various kinds, not just mesothelioma, in the asbestos workers that were employed in their shipyards. You can make all kinds of guesses. We just don't have the necessary data and probably we don't have the necessary personnel for this either. That's one of our biggest problems, I think, in this whole field - the shortage of trained personnel.

DR. CROCKER: We have a representative of NIOSH who might also be able to cast some light on this.

DR. JACOBSON: There's no point in speculating on what the next crisis carcinogen after vinyl chloride is. We're in it now, and that's arsenic. The Department of Labor hearings on arsenic were held last Friday. If I may pursue the question of mesothelioma, there is some controversial experimental data out of NCI that suggests that asbestos is not the only possible cause of mesothelioma but that fibrous glass, which is often considered a completely inert material, may also cause mesothelioma.

MR. WANDS: Dr. Jacobson knows as well as I do that Dr. Stanton's work at NCI is, indeed, controversial, but if you look at the data themselves, there are specific fiber sizes which under his experimental conditions do produce mesothelioma. There's no arguing with the data. There are arguments about the interpretation or the application of the data.

DR. JACOBSON: There are arguments about the data themselves. Paul Gross interprets the lesion as being a fibrosarcoma rather than a mesothelioma.

DR. STOKINGER: We had one other possible human carcinogen. I had the pleasure of having the visitation of the gentleman from Great Britain in my office recently, and they're putting the finger on antimony. The only problem in this matter is that the exposures are not pure. As everybody knows who has any experience with antimony, it is always contaminated with arsenic, so there is a question whether the arsenic is a cocarcinogen for antimony or is arsenic the culprit. The British are looking at the problem very closely because they have 17 deaths due to lung cancer alleged to have been caused by antimony. The 4 major producers of antimony in this country are getting together with their colleagues in Great Britain to investigate this problem.

DR. GROTH: I'd like to keep the record straight for the pathologists. The tumors induced by fibrous glass in Dr. Stanton's studies do look like fibrosarcomas but they also look like the human tumors which have been called mesotheliomas, some of which also look like fibrosarcomas.

DR. CROCKER: I think that's a note of clarification that this session should end on.

AMRL-TR-74-125

SESSION IV

CELLULAR TOXICOLOGY

Chairman

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AUTOMATION OF MICROSTEREOLOGY. THE DEVELOPMENT OF
A BIOLOGICAL TEST SYSTEM FOR EVALUATING THE PERFORMANCE
OF COMPUTER-CONTROLLER OPTICAL SCANNERS

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INTRODUCTION

A technical goal of the toxicological pathologists is to automate quantitative diagnostic microscopy techniques through the use of computer-controlled optical scanner methods. In order to accomplish this goal, a number of technical problems must be solved. Computer software must be developed to: (1) enable the computer program to identify specific cells and organelles from digitized representations of electron optical images; (2) direct the computer-controlled optical scanner to trace cell and organelle boundaries; and (3) abstract microstereology data from encoded segment lists that represent the coordinates of the boundaries of specific classes of cells and organelles. Boundary tracing within biological systems is of itself a complex and difficult problem because of the low contrast that is inherent in 60 nm thin sections of plastic embedded tissues, and variations and inconsistencies in gray levels at the perimeters of the structures of interest. This is in contradistinction to the phase boundaries within alloys and minerals which are generally quite easy to trace with computer-controlled optical scanners because the boundaries are well defined and of high contrast.

In order to develop computer software that will direct a computer-controlled optical scanner to trace biological boundaries, it is necessary to have a suitable biological test system that can be used to evaluate the efficiency and accuracy of programs and to calibrate the computer-controlled optical scanner. The purpose of this report is to describe such a test system. Human red blood cells were selected as the test material because they are reasonably uniform in size and shape, the cells are readily obtained by venipuncture from normal donors, and the red cell is reasonably electron dense as viewed by thin section transmission electron microscopy. Further, since major red cell dimensions including diameter, surface area and volume have been carefully

measured by several independent methods (Ponder, 1948; Canham and Burton, 1968; Evans and Fung, 1972) they are particularly useful as biological calibration standards against which new quantitative methods can be evaluated. This report also contains the first analysis of normal human red cell dimensions using microstereology methods.

MATERIALS AND METHODS

The RBC-Agar Embedding Technique

The following technique was devised to produce a biological standard consisting of randomly dispersed human red blood cells embedded in plastic and suitable for examination by thin section electron microscopy and analysis by microstereology techniques. Human RBC's were collected by venipuncture from five healthy male donors, heparinized, lightly centrifuged and the buffy coat was removed. Clinical data on the donors is listed in Table 1.

TABLE 1. CLINICAL DATA ON HUMAN RBC DONORS

<u>Donor</u>	<u>Age</u>	<u>HCT</u>	<u>RBC Count x 10⁶*</u>	<u>MCV (μm^3)*</u>
J. W.	17	42	5.75	72.5
R. W.	34	46	5.60	82.1
R. S.	23	45	5.57	80.8
M. C.	21	48	5.99	80.1
J. F.	<u>22</u>	<u>47</u>	<u>5.31</u>	<u>88.5</u>
Mean	23.6	45.6	5.65	80.8
± S.D.	± 6.77	± 2.30	± 0.255	± 5.78

*Determined with a Coulter Counter.

The packed RBCs were washed twice in isotonic phosphate-buffered saline pH 7.4 and were fixed for 1-1/2 hours at 4 C in 1% glutaraldehyde containing 6.70 gm NaCl/L, 0.675 gm KH_2PO_4 /L and 7.60 gm $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ /L. The cells were added dropwise to the fixative over a fifteen minute period until a ratio of fixative to cells of 10:1 was reached. After fixation, the cells were washed twice in isotonic phosphate buffer and resuspended in buffered saline to a final hematocrit of approximately 50%. Aliquots of cells from the suspensions were counted on a Coulter Counter. A freshly prepared 1% agar solution (prepared from "purified agar", DIFCO Corporation, Detroit, Michigan) was equilibrated at 48 C in a water bath. 0.5 ml of the agar was combined with 0.5 ml of the

RBC suspension and they were thoroughly mixed on a Vortex mixer. The RBC-agar mixtures were allowed to cool to 22 C over a 20-minute period and then placed in a refrigerator at 4 C for 45 minutes. With cooling, the RBC-agar mixture solidifies. The resulting RBC-agar gel was diced into 1.5 mm³ blocks. These blocks were post-fixed in 1% osmium tetroxide in phosphate buffer, pH 7.4, for one hour and dehydrated by serial passage through 70, 80, 95 and 100% ethanol solutions. The blocks were infiltrated with monomeric Epon 812 and the plastic was polymerized at 60 C. Thin sections were cut on diamond knives and the sections were stained with uranyl acetate and lead citrate.

Agar-Shrinkage Artifacts

A potential source of dimensional distortion with the RBC-agar technique is shrinkage of the specimen that might be introduced during polymerization of agar. Shrinkage was determined experimentally to be less than 0.6% which is regarded as trivial.

Electron Microscopy for Microstereology Analysis

Thin sections were mounted on 400 mesh copper grids and photographed in a Philips EM 300 electron microscope according to the systematic random sampling procedures as summarized by Weibel (1969). In brief, large sections of blocks were cut so that individual sections covered many grid squares. The upper left hand corner of each of 10-32 consecutive grid squares were photographed on Kodak SO 410 35 mm film at original magnification of 1800X. Electron microscope magnifications were precisely calibrated before each set of electron micrographs with a 28,800 line/inch calibration grating (Ladd, Burlington, Vt.). 35 mm film strips containing the electron micrographs from individual donors were spliced together and coded for purposes of identification. Included on the final test film are 10 electron micrographs of thin sections of five RBC-agar blocks from each of five donors. Thus, the spliced test film contains a total of 250 electron micrographs of human red blood cells.

Microstereology Analysis of RBC's by Hand Counting

Microstereology analysis of red cells was carried out using a Weibel microprojection morphometrics table and a multipurpose test lattice system (Weibel, 1969). The multipurpose test lattice system consists of a triangular lattice of 168 test points with every other point connected to its following neighbor by a line. These lines, in aggregate, are defined as the "test line" (L_T). In order to perform microstereology analysis of red cells, electron micrographs are individually projected onto the screen of the Weibel microprojection table. The multipurpose test system of lines is printed on the table screen.

With the test system, it is possible to determine the mean corpuscular volume and mean surface area for a population of RBC's using hand counting methods. Figure 1 illustrates how microstereology data is obtained with the Weibel multipurpose test lattice. The lattice is superimposed over an electron micrograph of a thin section of randomly oriented RBCs. The ends of each test line represent "test points" (e.g. 2 points per test line). The structure under each test point is identified and these are recorded on a differential counter. Next, intercepts are tallied. An intercept is defined as a point where an element of the test line (L_T) crosses a cell boundary, as illustrated in Figure 1.

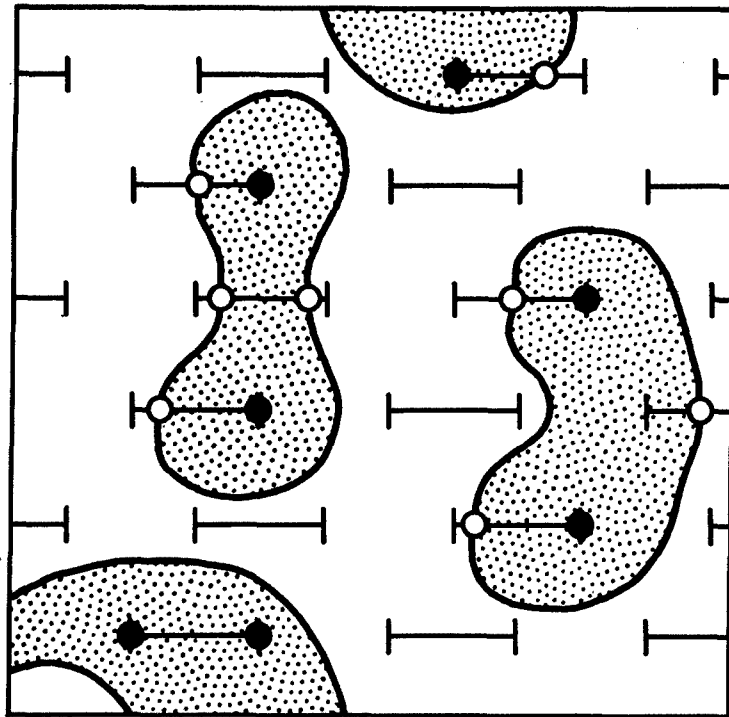


Figure 1. Diagrammatic representation of part of the multipurpose test lattice system, shown superimposed over a drawing of sectioned RBCs (stippled profiles). Test points over RBCs (P_{RBC}) are represented as closed circles. Intercepts (I_{RBC}) are points at which the test line crosses the cell surface and these are shown as open circles.

Determination of Mean Cell Volume

For each donor, the mean RBC cell volumes and RBC surface areas were measured by microstereology methods. The number of RBCs per unit volume in the RBC-agar blocks were calculated. Partial volumes (e.g. hematocrits) for RBC in the final plastic blocks were calculated according to the following formulae:

$$V_{V_{RBC}} \text{ (HCT)} = \frac{V_{RBC}}{V_T} = \frac{P_{RBC}}{P_T} \quad (1)$$

where $V_{V_{RBC}}$ is the volume fraction of the cell block that is occupied by RBCs, V_{RBC} is the volume of RBCs in the blocks, V_T is the total volume of the block, P_{RBC} is the number of test points over RBCs, P_T is the total number of test points (e.g. 168 per electron micrograph). The mean corpuscular volume (MCV) of red blood cells is:

$$MCV = \frac{V_{V_{RBC}} \cdot \text{mm}^3}{N \cdot D} \quad (2)$$

where N is the number of RBCs per mm^3 and D is a dilution factor representing the extent of dilution of the red cell solution in the final RBC-agar mixture ($D \approx 0.5$).

RBC Surface Area Measurements

The total surface area of the RBCs is determined by the formula:

$$SV = 2 \cdot I / L_T \quad (3)$$

where I represents the number of intercepts at RBC surfaces and L_T is the total length of the lines in the multipurpose test line system (Weibel, 1969).

RESULTS

Electron Microscopy of Human Red Cells

Figure 2 shows prints of consecutive electron micrographs of human RBCs that were recorded on 35 mm film. The cells are of relatively high contrast and are suitable for microstereology analysis by a computer-controlled optical scanner.



Figure 2. A-H: Consecutive electron micrographs from neighboring fields of a thin section of randomly oriented human RBCs in an RBC-agar gel. Magnification X2000. I: Electron micrograph of the germanium shadowed carbon replica that is used to calibrate the electron microscope magnification for each film strip. Calibration grating spacing is 28,800 lines per inch.

Determination of the Representative Sample

In applying microstereology methods to specific problems in biology, such as the determination of red cell dimensions, it must be determined how many frames (electron micrographs) should be pooled to achieve a representative sample. This is important because: (1) the specimens may be heterogeneous; and (2) a single electron micrograph represents a statistically inadequate sample. For the purposes of this study, the acceptable mean value for cell volume in each RBC-agar block was set at $\pm 10\%$ of the value derived from pooled counts on large numbers of test frames (i.e. > 25 frames). Figure 3 shows data obtained from counts on 32 consecutive frames from a single thin section of human RBCs. The curve shows that the cumulative means are within the $\pm 10\%$ limits when six frames are analyzed and they remain within the $\pm 10\%$ range with the addition of subsequent tallies.

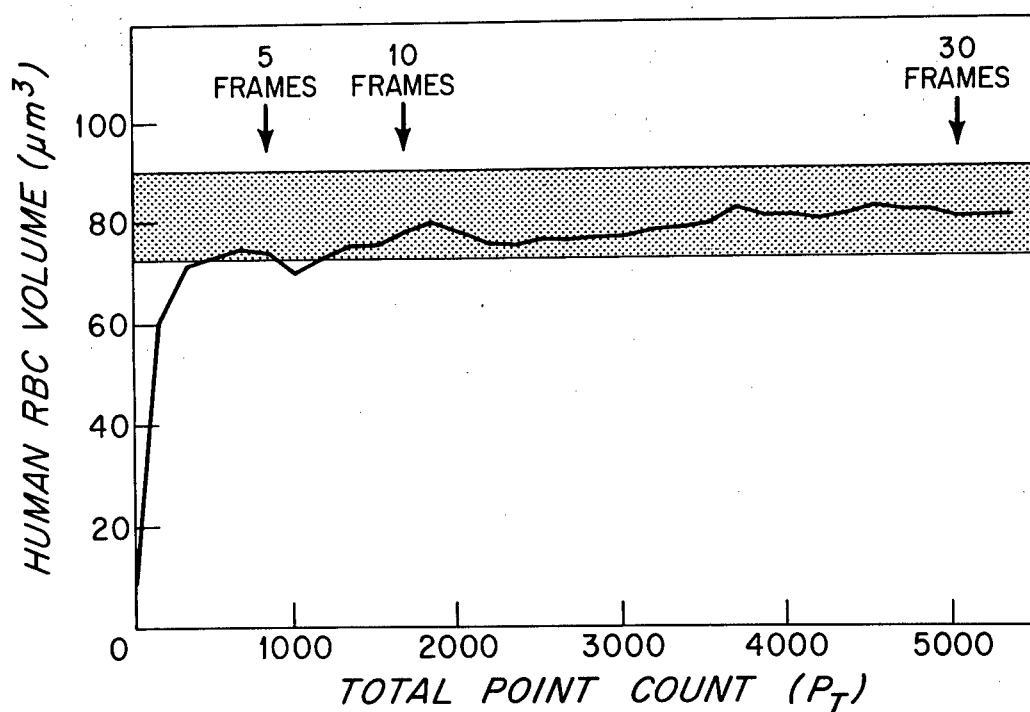


Figure 3. Cumulative mean values for the measurement of RBC mean corpuscular volume by microstereology hand counting methods. 168 test points were tallied for each electron micrograph. The mean value obtained from counts on 10 micrographs (1680 test points) is essentially the same as the cumulative mean from 30 frames. The stippled zone represents $\pm 10\%$ of the final mean value.

Table 2 shows the surface area and MCV values for five normal human donors, as determined by microstereology methods. The mean MCV value for the five donors was $79.6 \mu\text{m}^3$. Mean cell surface areas ranged from $122.8 \mu\text{m}^2$ to $133.9 \mu\text{m}^2$ and the mean value for five normal human male donors was $130.1 \mu\text{m}^2$. MCV values were compared with MCV values for the same donors as measured with the Coulter counter (Table 1). Values obtained by the two methods are not significantly different (Students t-test, $P < .01$).

TABLE 2. MICROSTEREOLOGY DATA ON HUMAN RBC'S

<u>Donor</u>	<u>HCT</u>	<u>Surface Area (μm^2)</u>	<u>MCV (μm^3)</u>	<u>S/V Ratio</u>
J. W.	42	133.4	76.9	1.74
R. W.	43	133.7	75.0	1.78
R. S.	46	133.9	81.8	1.63
M. C.	47	122.8	76.8	1.41
J. F.	<u>47</u>	<u>122.8</u>	<u>87.4</u>	<u>1.41</u>
Mean	45.00	130.14	79.58	1.628
\pm S. D.	± 2.345	± 5.045	± 5.049	$\pm .1462$

DISCUSSION

In this report, a method is described that can be used to prepare a biological test object, the human RBC, for thin section electron microscopy. Red cells are randomly and relatively uniformly distributed in the RBC-agar gels prepared according to this method. The cells are of reasonably high contrast and preliminary testing indicates that these films of RBC-agar blocks are suitable for use in a computer-controlled optical scanner, thus satisfying our technical objective.

The microstereology data human RBCs that were developed in the course of this study are of considerable general interest. Estimates of RBC MCVs and surface areas are of considerable importance since these values are used in studies dealing with a broad spectrum of problems in cell biology, biophysics and biochemistry (Weinstein, 1974). Many investigators have attempted to determine these RBC dimensions by other methods and some of these results are summarized in Table 3.

TABLE 3. ESTIMATES OF HUMAN RED CELL MAJOR DIMENSIONS
BY DIRECT MEASUREMENTS

<u>Surface Area</u>	<u>Volume</u>	<u>Reference</u>
($\mu\text{m}^2 \pm \text{S.D.}$)	($\mu\text{m}^3 \pm \text{S.D.}$)	
138.1 \pm 17.4	107.0 \pm 16.8	Canham and Burton, 1968
134.0 \pm 4.9	82.0 \pm 4.9	Houchin et al., 1958
135.0 \pm 16	94.0 \pm 14	Evans and Fung, 1972

These previous studies have relied upon the light microscope for measurements on RBCs. In the current study, we have employed electron microscopy techniques to determine RBC dimensions. Although this approach has the advantage of the higher resolution of the electron microscope, there may be certain disadvantages. Specimens must be chemically fixed, dehydrated through graded ethanol solutions, embedded in plastic and thin sectioned. These procedures may introduce dimensional distortions (Weibel, 1969). Dimensional distortion from these potential sources of artifacts might account for our estimate of RBC surface area, $130.1 \mu\text{m}^2$, which is 2.9 to 6.1% lower than the value estimated by other methods. It is also entirely possible that our value is more accurate than that obtained by other methods. This question can only be resolved by a systematic study of the artifacts of thin section electron microscopy preparative techniques. It is noteworthy that the microstereology approach has certain advantages over conventional quantitative microscopy techniques. In particular, the method is shape independent thus making possible measurements on toxically injured cells of various shapes.

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USE OF COMPUTERS IN DIAGNOSTIC PATHOLOGY IN LABORATORY MEDICINE

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INTRODUCTION

Among physicians the role of computers in medicine is still very much a matter of debate. Their use in billing, and accounting and by insurance carriers is accepted as a necessary evil to satisfy the ever increasing demand for figures which large organizations need to function. Intensive Care units and chemistry laboratories and some other applications have brought the computer to the bedside and the laboratory in a small but growing number of institutions.

What about a pathology laboratory? As part of its function it must perform a great deal of accurate accounting. Specimens must be logged in; their flow through the laboratory must be traced, and the results must be reported within prescribed dead-lines; the accuracy of identification and reporting must be constantly monitored; charges for all procedures must usually be detailed and long term access to pathology reports must be possible for any patient. Retrieval of all previous reports should routinely be done whenever a new specimen from the same patient is to be examined. Finally, ready retrieval for teaching and research purposes of pathology specimens specified by type is desirable. All of this kind of book-keeping is exactly what computerized information systems are designed to do efficiently. With small systems, such as the MUMPS (Digital Equipment Corporation, 1972) system, their cost effective scale may well be small enough to fit the needs of pathology laboratories serving teaching hospitals with as few as 400 or 500 beds. Pathologists have been in the forefront of coding medical terms suitable for computer processing, but in spite of the wide use of the SNOP codes (College of American Pathologists, 1969), computers are not yet used in pathology laboratories, performing the kind of functions outlined above. However, the computer technology and software for this application exists right now.

IMAGE ANALYSIS

Further in the future is the computer to help in the microscopic or electron microscope examination of tissue. It is a far different problem, which impinges on a process or skill for which people are extremely well qualified. Our visual sense is probably our most developed one. It is not for nothing that we say "one picture is worth a thousand words." In fact, it takes a computer as much memory to store a 35 mm transparency as it would take to store all the letters from about 300 printed pages. Yet there are visual tasks in pathology which appear to be relatively simple, repetitive and extremely time consuming. If any of them also require quantitative observations, such as measuring volumes of cells or organelles or the areas of cell membranes, computerized systems may offer attractive solutions.

In order to appreciate the potentialities and limitations of computers in image analysis it may be appropriate to describe briefly how an image is typically dealt with by a computer. For more details, a review recently published by one of the authors (Neurath, 1974), and the special issue of the Journal of Histochemistry and Cytochemistry (containing papers from the Engineering Foundation Conference on Automatic Cytology, 1974) which contains descriptions of on-going work from many laboratories may be consulted.

Let us assume that the image to be analyzed is on one frame of a black and white negative or positive film. This film frame can be centered and placed in the correct focal plane of a scanning device by hand. The alternative, to analyze images of tissue directly from microscope slides, requires finding a suitable area on the slide and focussing it. This presents additional interesting and difficult problems for automation, some of which are discussed in the articles referred to above. However, the automatic finding and focussing systems constitute almost separate problems from the central one we want to describe, namely how a computer can analyze a picture, and will therefore be ignored here.

Instead we start with the need to scan the focussed image on film. This means it must be broken up into many separate dots: For each dot the density of the film must be measured; and the measurement must be converted to a number (digitized) which can be placed into the computer's memory, together with the address of the dot. A number of technical problems and options to solve these tasks exist. These include scanner speed, resolution, spatial linearity, grey scale range and linearity, noise, the possibility (or lack) of random access to parts of the image, the choice of dual or split beam reference systems versus single beam systems, and the role of low speed magnetic (disk) memory to hold a large number of digitized image dots which the computer cannot use effectively without transferring large blocks of them into high speed core memory for actual processing.

Currently the typical performance ranges of scanners are to digitize 50,000 to 500,000 dots per second, with 16 to 128 grey levels distinguishable above noise, with 32,000 to 640,000 dots per frame that can be scanned. Low frequency non-uniformity over the whole frame is generally no better than $\pm 5\%$, and the maximum amount of fast memory that is practical to use for storing a digitized portion of a picture is 128,000 bytes of 8 binary bits each, at the most, equivalent to 128,000 dots at 64 grey levels. For comparison it should be pointed out that a 35 mm frame of fine grain black and white film stores approximately 2 million dots at 128 distinguishable levels of density, or 30 times as much, while a home TV screen resolves about 260,000 points to 10 levels.

Fortunately, certain interesting features of a picture generally represent much less information which can then be expressed in a few numbers. These are the parameters of the object or objects in the image. Typical ones are area, density, color, diameter, length, circumference, moments of various dimensions, such as the moment of area, shape descriptors such as eccentricity of an ellipse or the number of concave and convex parts of a boundary, and texture measures such as the coefficients of a two dimensional Fourier representation of the density variations. Using such parameters, human chromosomes have been analyzed successfully (Selles, 1974) and more than a dozen types of white cells can be differentiated automatically (Brenner, 1974).

BOUNDARY DETERMINATIONS

A simple, illustrative example is the quantitative measurement of red blood cell size, specifically that of measuring the relative volume of cells embedded in a matrix. As DeHoff and Rhines (1968) have shown, the relative volume $V_v = V/L^3$ equals the expected value of the relative area $A_a(Z) = A(Z)/L^2$ of cells in planes, averaged over all planes normal to Z in the test cube of side L . Therefore one only needs to measure the relative area of cells to matrix in a number of planes. One way to do this by computation is to first identify the cell boundaries in the plane. A way to determine a boundary of an object and thus separate it from the background is a basic procedure useful in many image analysis problems. The human eye is very well adapted to find boundaries, and people in fact tend to analyze images by identifying or creating boundaries. One way for the computer to perform this function depends on the assumption that the object sought is darker (or lighter) than the matrix. One then checks through every point in the image, one line at a time, until one finds a point darker than a pre-selected threshold. This is the first boundary point. Starting from it, one checks its 8 adjacent raster points in sequence to find the next boundary point and so on, tracing the boundary in for

example, a clockwise direction, until one returns to the original starting point, having circumscribed a cell. One then takes up the search again. Two complications must be taken into account. One is the need to avoid finding the same object again after running the line by line scan.

This can be avoided by for instance setting the grey level of the boundary points found to a special level, such as zero or the maximum, not otherwise used. Then points falling between two previously found labelled boundary points on the same boundary do not indicate new objects. Tangential points must be treated specially. A second complication is the existence of matrix areas interior to cells. These must be looked for as well by scanning the interior of a cell.

REQUIRED SOPHISTICATIONS

Unfortunately these fairly straightforward procedures do not, in general, give the expected results. The boundary obtained by the computer does not always coincide with the boundary an observer would draw, particularly if it is not sharp to begin with. The reason is that even though the cell may on the whole always be appreciably darker than the matrix, a fixed threshold does not describe this fact adequately: (i) There may be local irregularities at the boundary which an observer knows to ignore. (ii) Some parts of the matrix or the cell may be darker than others, effectively shifting the threshold at a broad or wide cell boundary as shown in Figure 1. At A the true threshold is probably at level 25, while at B it is at level 20. (iii) The sensitivity of the scanner or the exposure of the film may vary from one side of the frame to the other in some fashion, making both cell and background appear to be lighter than they are in some areas of the frame and darker in others. This is called shading. Its effects on the threshold of the real cell boundary would be similar to those depicted in Figure 1, unless the shading is so severe as to change cell interior levels to matrix levels or vice versa, making any fixed threshold between 10 and 30 unsuitable.

There are two reasons why an observer is not affected by these three factors. He may know that the cell boundary is fairly smooth and round and continuous and even if a break appears at some point, that this is an artifact of preparation, not representative of the living cell. He also does not recognize the boundary demarcation on the basis of a fixed threshold, but uses the local maximum gradient normal to the boundary which is independent of variations in local darkness and of shading.

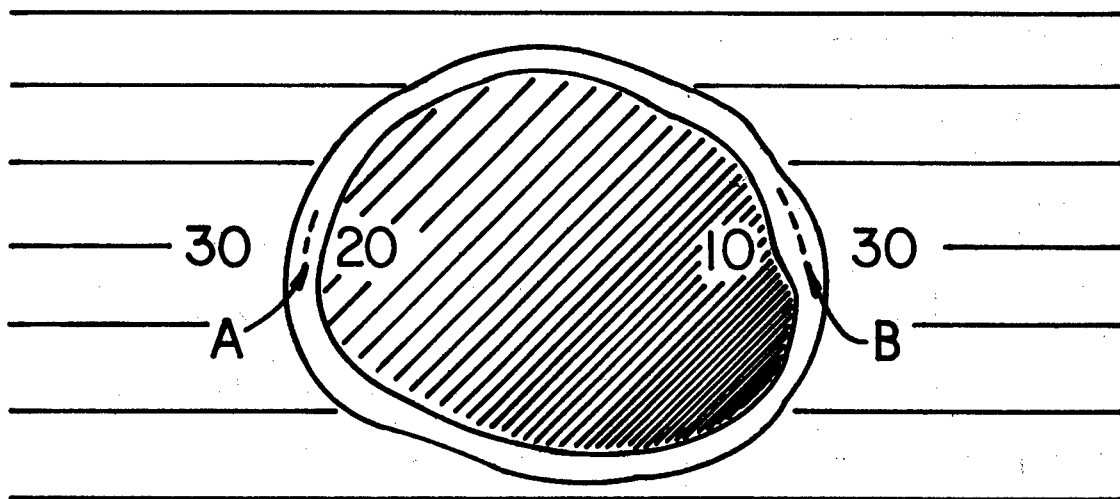


Figure 1. A cell and its boundary layer. Near A the cell is lighter (transmission level $L=20$) than at B ($L=10$) while the surrounding matrix is constant ($L=30$).

Both of these human observer characteristics can, within limits, be incorporated as parts of computer boundary finding programs, but only at the expense of a considerable amount of additional computation. We have made extensive use of a program that introduces the local gradient to modify all the grey levels so as to allow finding boundaries by their gradient. We apply a transformation to all points in the frame before we look for boundaries with a constant threshold. The transformation consists of replacing the grey level of every point with the algebraic sum of the weighted grey levels of neighboring points within a radius of several raster points. In our most commonly used scheme, only 13 out of the 49 neighboring points have non zero weights for the sake of speed of execution. The weights are shown in Figure 2. When there is no gradient, this transformation changes all points to zero. When a gradient is encountered, between a lower and higher plateau, then points on the gradient near the lower one are made negative, those near the higher plateau are made positive. Thus a boundary finding program using almost only positive threshold will not only find the boundary, but the boundary will be much sharpened if the original boundary fuzziness is of the order of 7 raster points wide. This is shown along one dimension in Figure 3 which shows a cross section of the grey levels at a cell boundary region before and after the transformation.

			-1			
	-1				-1	
			1			
-1		1	4	1		-1
			1			
	-1				-1	
			-1			

Figure 2. The transformed grey level of the point P is computed from its original level and the level of 48 neighboring points, each level being multiplied by the weighting factor shown. The weighting factors of the blank squares are zero.

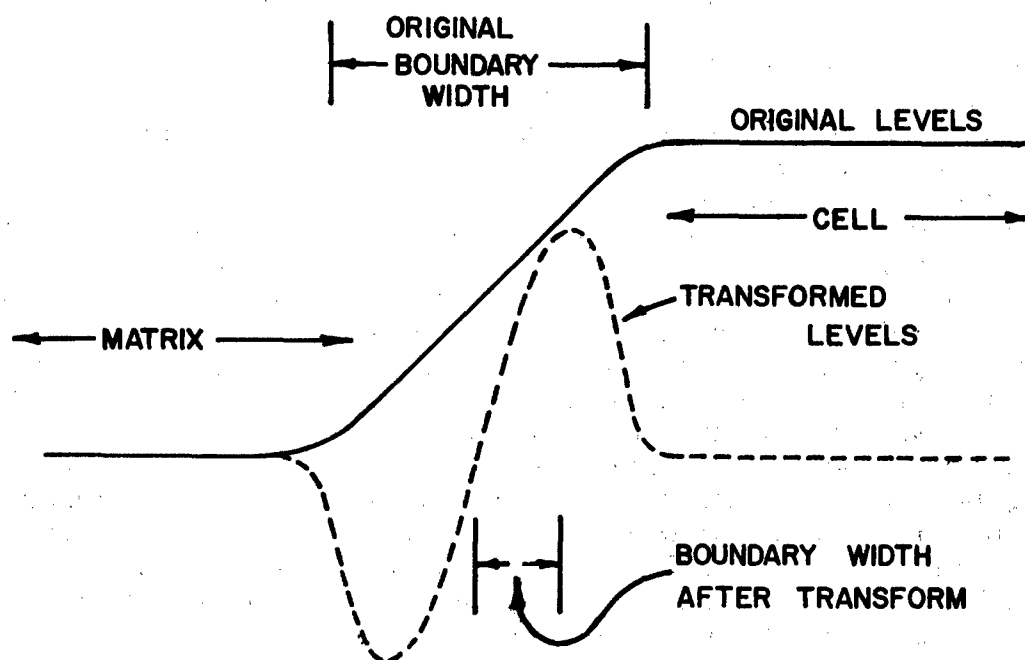


Figure 3. This illustrates the decrease in the boundary indeterminacy if any threshold above matrix level (or zero level for the transformed image) is permitted, when the transformation shown in Figure 2 is used.

The error in using a fixed threshold is much decreased for the transformed profile. The transformation also tends to smooth out local irregularities which are small compared to the 7 x 7 raster unit square used. As can be expected from a derivative, the local grey level "noise" extending over an area equal to or greater than 7 x 7 raster points is accentuated rather than smoothed. Also, if the real cell boundary is in fact sharply curved, it will be rounded off by this program. This will for instance reduce the size of small sections of cells. This latter problem is not relevant when outlining red cells are about equally large. However, it is a problem when measuring cell cross sectional areas from EM sections of cells at arbitrary depths of the cell, as some sections will be quite small in diameter. The matter of treating small cell sections differently from large ones requires an iterative procedure of some sort. That is, we first have to close a boundary around an object by a fixed rule before we can know that the object is small and requires a rule modified for small objects.

If the boundary is sharp compared to the raster spacing used, but overall shading is a serious problem, then the threshold, instead of being a fixed one, can be set differently in each of any number of subdivisions of the whole frame, for example, in 24 squares. The setting of the threshold itself in that case must probably be done by the program to save time, while when selecting a single threshold per frame, operator interaction is a possible option.

At this point, let us list the procedures we have selected in the order in which they might be executed. It is well to remember that, although typical, other ways of dealing with these problems are certainly possible.

1. Obtain good photographic negatives (minimize shading as much as possible).
2. Center frame and focus scanner.
3. Divide frame into 24 squares. Take each square, obtain a level histogram and determine the threshold in each.
4. Start an object finding and object tracing routine using the proper threshold in each square.
5. When an object touches the boundary of a square, special consideration must be given to its continuation into the next square(s) if these have different thresholds.
6. Re-trace boundaries of small objects.

7. Check all objects found for size and shape. Scratches in the film or pinholes have characteristic dimensions incompatible with cell sections. Similarly, any object larger than the largest possible cell section must be in error.

AREA AND CIRCUMFERENCE MEASURES

At the end of these procedures, a valid set of cell boundaries to obtain the surface and volume fractions by the equations stated before should be at hand, were it not for the digitization problem. Substituting discrete points for a continuous picture requires careful definition of what we mean by area and circumference. The area is perhaps the simpler of the two. If the digitization raster is very fine (i. e. of very high resolution), then the number of points darker than threshold times the area per raster point will approach the area of the object and can be used as the definition of the area. However, if, to save computer time, we use a rather coarse raster, then using only the points definitely above threshold will underestimate the enclosed area, and the underestimate will be more severe the larger the ratio of circumference to area is. Basically, an area about equal to half the area of the circumference times a raster width must be added to the cell area.

The cell section circumference is not approached more accurately by increasing the raster resolution, as can be seen by reference to the sketches in Figure 4.

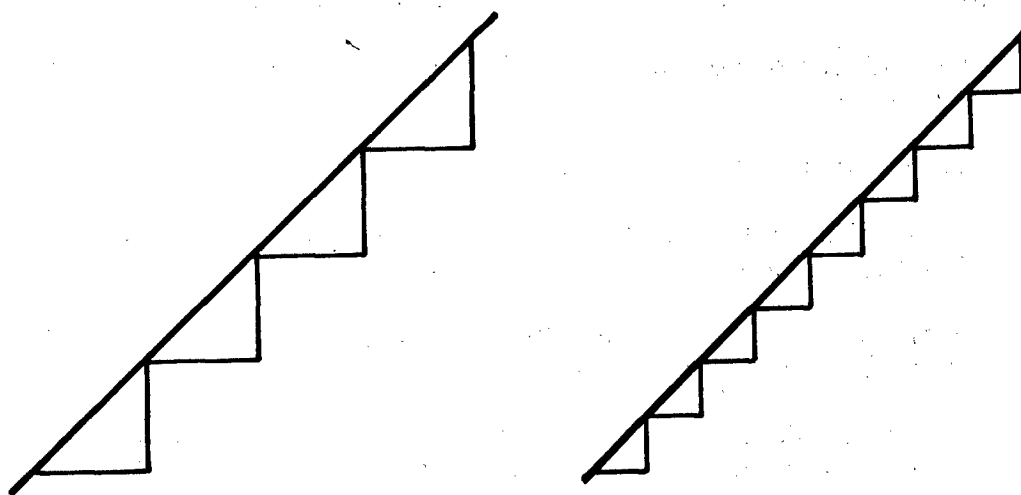


Figure 4. Whether the raster spacing is large or small, the length of the stepped segments connecting nearest neighbor raster points is always $\sqrt{2}$ times larger than that of the heavy line which these steps approximate.

No simple correction factor relates the lengths of a curved line to the number of raster points just inside the boundary or even to the stepped line traced out by connecting raster points with any of their nearest (and even next nearest) neighbors as shown in Figure 5. For instance, the circumference of a rectangle oriented along the axes of the raster would be accurately represented, while the curve shown in Figure 5 is shorter than the straight line segments, by an amount determined by the shape of the curve and its orientation to the raster axis. For given sizes and shapes of fairly round objects, an empirical correction factor could be applied. However, to obtain a more accurate answer good for any shaped object, one must compute a smoothed circumferential curve so that for instance the line segments connecting the smoothed points will always form angles smaller than $\pm 45^\circ$ with each other. The smoothing algorithm together with the raster spacing will of course determine the smallest radius of curvature of the cell boundary that can be approximated. The algorithm could of course use half or quarter raster spaces to compute a smoothed curve.

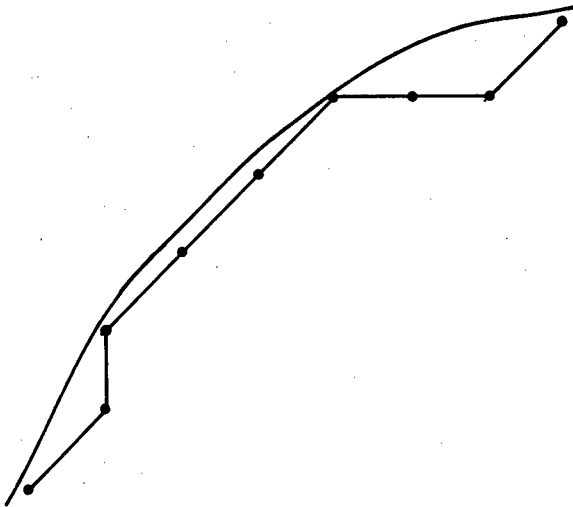


Figure 5. Curved boundary represented by straight line segments of raster length 1 and $\sqrt{2}$ connecting nearest and next nearest neighbor raster points just inside of the boundary.

For instance, the length of the curve AB which is a straight line shown in Figure 6 is $\sqrt{5^2 + 1^2} = 5.10$ raster units. The length of the raster segments using nearest neighbors is 6; including next nearest neighbors without smoothing it is 5.41 or about 6% higher than the true length. The lengths of segments smoothed by interpolating to half a raster spacing when the angle is 45° is 5.24 or about 3% longer than the curve AB. Clearly, considerable smoothing is required to be accurate to better than 2%. To achieve that kind of accuracy, the circumference must also be corrected for the boundary thickness itself. As in the case of the area, if our boundary points are always the points within the cell, the true boundary is approximately $1/2$ a raster spacing outside those points. For a circle, an increase in diameter of 1% will increase its circumference proportionately; similar differences would be produced in the perimeter of other shapes, and must be corrected for.

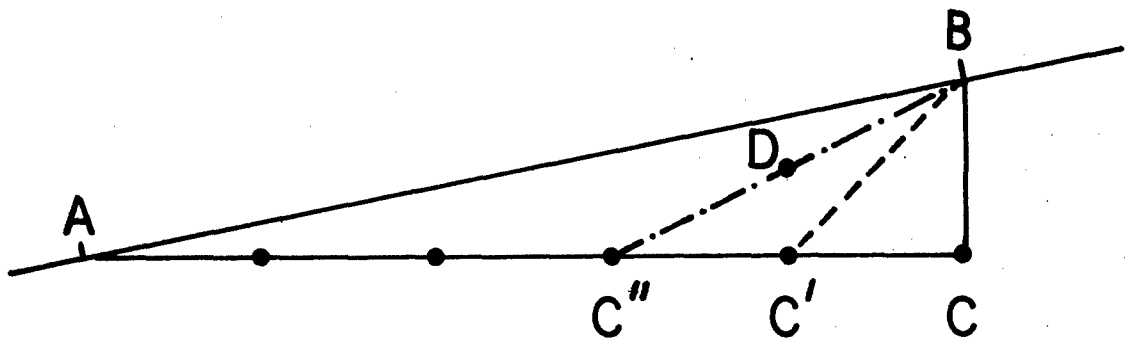


Figure 6. The length of the curve AB approximated by segments is 6 units along ACB; 5.41 units along AC'B; and 5.24 units along AC''DB. The actual length of AB is 5.1 units. The point D represents an interpolation going from C'' to B, to be invoked because angle BC'C is too large to represent a "smooth" curve.

The corrections for obtaining the boundary to 1 or 2% accuracy converts a simple counting of boundary points to a considerably more time consuming calculation involving checking the angle between every neighboring point and making a final adjustment that depends on the ratio of the raster size to boundary length. Even with these programs, reliable operation of these programs requires good negatives which are not only evenly exposed and have the correct range of densities which the program expects, but which must not contain artifacts the program is not equipped to handle.

TYPICAL RESULTS FOR AREAS AND CIRCUMFERENCES

With these provisions the computer system, using the sophistications listed, can handle the problem illustrated at relatively low cost and high speed and accuracy if large volumes of pictures are handled in a given period of time. Of course the problem discussed is a rather trivial one for people, and one for which morphometric sampling methods are also able to give results of adequate accuracy with only a moderate effort of counting intercepts.

If all of the computational sophistications are not followed, the computer results show appreciable errors. This is best illustrated by some of our preliminary results which were done the quick and easy way. We used a relatively coarse digitizing raster, with points separated by approximately 0.2 microns, i. e. about 40 points along a 7μ cell diameter. The boundary was determined by a threshold adjusted for each of 24 squares per frame. The area was only corrected by the addition of 0.56 times the area of the number of boundary points and the length was based on a simple boundary point count, by assuming it to be equal to the number of such points multiplied by $1/2(\sqrt{2} + 1)$ raster spacings. Typical data obtained are given in Table 1 below. Relative cell areas are obtained from boundary lengths of the sections, cell volumes from the cell section areas.

TABLE 1. PERCENT DISCREPANCIES D IN CALCULATED RELATIVE AREA A AND VOLUME V BETWEEN MANUAL AND COMPUTER MEASUREMENTS

Sample No.	1	2	3	4	5	6	7	Ave.
%D _A	4	-1	9	9	-14	14	-12	+0.5
%D _V	-11	-9	-13	-6	-26	4	-18	-14.0

These initial results seemed to show systematic agreement and discrepancy, both perhaps fortuitous. As the manual measurements of cell parameters are also subject to error, additional measurements using the same simple computational techniques to illustrate various computational problems were made on clear and sharp images of standard reference circles. One set illustrates the effect of raster spacing relative to circle diameter and is shown in Table 2.

TABLE 2. PERCENT DISCREPANCIES, D, BETWEEN TRUE AREA AND COMPUTED AREAS AND CIRCUMFERENCES OF STANDARD CIRCLES

<u>Diameter of Circle</u>	<u>4.5 mm</u>				<u>6.6 mm</u>		
Approx. Percent of Raster Spacings in Diameter	120	60	40	30	90	60	45
% D _{area}	-1	-2	-2	-5	-3	-4	-5
% D _{circumference}	7	7	8	5	7	7	6

As can be seen, a relatively fine raster spacing decreases the error in the area but not in the circumference, and this agrees with our discussion of the factors involved.

WHITE CELL DIFFERENTIATION AND CHROMOSOME ANALYSIS RESULTS

Although the difficulties of just obtaining correct area and circumference may appear formidable, the more complicated procedures of white blood cell differentiation and of chromosome analysis can in fact be handled successfully by extensions of these techniques coupled with pattern recognition algorithms. These algorithms use the measurements obtained, such as cell area, diameter, color and others to classify the cell or chromosome. The references previously cited give further details on methods and results. For the differential white blood cell problem, several commercial companies are building and marketing computer operated microscope systems which, if successful, promise to make a major impact on the hematology work of clinical laboratories.

CONCLUSIONS

The ever increasing need for accurate, large scale laboratory measurements of microscopic specimens is expected to stimulate the application of computer techniques to any routine which is or becomes widely used. To facilitate such applications in future, research in computerized measuring techniques and pattern recognition algorithms, as well as in hardware for scanning and computing, needs to be performed. One way to make sure that this research gets done, and that its results will be readily usable in the pathology laboratory is to have a few pathologists become actively and intimately involved with it. It is hoped that this brief exposition has not only laid to rest any fears that computers are about to replace pathologists at the microscope, but also inspired some to examine more deeply what it is that our eyes observe and test it against the strict requirements of the simple-minded computer.

ACKNOWLEDGEMENT

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AEROSOL PARTICLE SIZE AS A FACTOR IN PULMONARY TOXICITY*

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"Indeed, for a given magnitude of atmospheric exposure to a potentially toxic particulate contaminant, the resulting hazard can range all the way from an insignificant level to one of great danger, depending upon the size of the inhaled particles and other factors that determine their fate in the respiratory system."

Theodore Hatch and Paul Gross from the introduction in Pulmonary Deposition and Retention of Inhaled Aerosols, Academic Press, N. Y. 1974, pg. 2.

INTRODUCTION

The respiratory tract is both a portal of entry and a target for environmental air pollutants. In an industrial society vast numbers of people are exposed occupationally and more generally, environmentally, to a variety of dusts, fumes and other aerosols which may produce lung disease. Particulate toxic agents include asbestos, silica, metal fumes, infectious agents, acid mists, fibrous glass, and in the nuclear industry, radioactive aerosols. Important considerations in assessing inhalation hazards include the biological status of the exposed individual and the chemical and physical characteristics of the aerosol. Factors related to particle size that influence the toxicity of inhaled aerosols in humans include mass per particle, aerodynamic behavior, rate of dissolution in the lung, efficiency of uptake by macrophages, and the ability of particles to penetrate biological membranes.

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AEROSOLS

An aerosol is a relatively stable suspension of small, solid particles or liquid droplets in a gas. Only a smooth, spherical particle or droplet can be conveniently described by a unique geometric diameter. Since aerosols of solids rarely consist of smooth, spherical particles, conventions for particle diameters are defined which are usually based upon available measurement techniques. For example, the size of a particle may be described in terms of its projected area diameter, some defined as the geometric diameter of a circle which has the same area as the two-dimensional outline of the particle lying on a collection surface. Other conventions for describing physical size can be based on measurements of scattered light, surface area, electrical mobility or other physical or chemical phenomena. Methods for physical sizing of aerosols have been discussed by Raabe (1970) and by Mercer (1973).

Because important inertial properties of particles, such as settling speed or ability to turn corners in a moving air stream, depend on factors such as density and shape in addition to physical size, it is often useful to describe particles in terms of an aerodynamic (equivalent) diameter. The aerodynamic (equivalent) diameter which is usually used in inhalation toxicology is the geometric diameter of a spherical particle of unit density material ($\rho = 1 \text{ gm/cm}^3$) which has the same settling velocity (in still air) as the particle being described. Two particles having markedly different densities or shapes may vary considerably in physical diameter but have the same aerodynamic diameter.

For particles larger than about 0.5 micrometer (μm) in physical diameter where inertial and gravitational forces dominate particle motion, the aerodynamic diameter can be used to predict particle deposition in the respiratory tract. Below about 0.5 μm the particle size is approaching the mean-free-path between collisions of air molecules, and diffusional forces tend to dominate the particle motion and the physical diameter of the particle correlates more closely with aerodynamic behavior, and should be used when considering particle motion.

Since individual particles in a given aerosol usually vary widely in size, statistical descriptions are often used to describe aerosols. For example, aerosol size distributions may be described by a mean physical or aerodynamic diameter and the associated standard deviation (or by a median diameter and geometric standard deviation).

INHALED PARTICLE DEPOSITION AND CLEARANCE

Inhaled particles may deposit on the various surfaces of the respiratory tract. The Task Group on Lung Dynamics of the International Commission on Radiological Protection has proposed a general model useful in estimating the potential hazards associated with inhaled aerosols (Task Group on Lung Dynamics, 1966). This model includes estimates of both the fractional deposition of inhaled particles with respect to aerodynamic size, and clearance of deposited particles from the respiratory tract with respect to deposition region and basic particle properties. The model divides the respiratory tract into three regions based upon anatomical features and particle deposition and clearance phenomena. The regions, called (a) the nasopharynx (NP), (b) the tracheobronchial region (TB) and (c) the pulmonary or parenchymal region (P), are referred to in Figure 1.

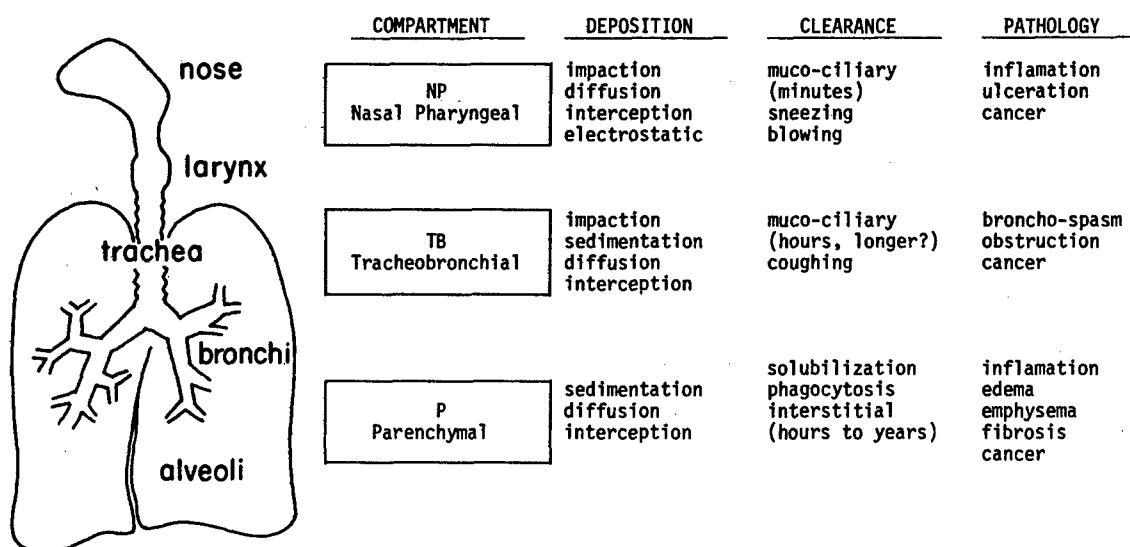


Figure 1. Compartmental model of the respiratory tract as used by the Task Group on Lung Dynamics of the ICRP (1966) with the airways from nose to larynx in the NP (nasopharyngeal) region, the trachea and ciliated bronchi and bronchioles in the (tracheobronchial) region and the nonciliated bronchioles, alveolar ducts, alveolar sacs, atria and alveoli in the P (parenchymal or pulmonary) region. The types of particle deposition, clearance and potential pathology are summarized for each.

The nasopharynx (NP) begins at the anterior nares and includes the respiratory airway down to the level of the larynx. Particle deposition in this region is primarily limited to the larger particles whose inertial properties cause impaction in the nasal passages or entrapment by nasal hairs. Two pathways, both having a half-time of 4 minutes, are used by the Task Group to describe the clearance of particles which deposit in the NP compartment. The first describes uptake of relatively soluble material into the blood, while the second represents physical clearance by muco-ciliary transport to the throat for subsequent swallowing.

Experimental data indicate that the anterior one-third of the nose, where 80% of 7 μm particles deposit, does not clear except by blowing, wiping or other extrinsic means (Walsh, 1970; Proctor, 1971) and effective removal of insoluble particles may require one to two days. The posterior portions of the nose have mucociliary clearance, with clearance half-times of about 6-7 hour (Morrow, 1972).

The tracheobronchial region (TB) begins at the larynx and includes the trachea and the ciliated bronchial airways down to and including the terminal bronchioles. A relatively small fraction of all sizes of particles which pass through the NP region will deposit in the tracheobronchial region. The mechanisms of inertial impaction at bifurcations, sedimentation and, for small particles, Brownian diffusion cause TB deposition. Interception can be an important deposition mechanism for fibrous dusts. In mouth breathing of aerosols, such as in cigarette smoking, the benefits of the collection of larger particles in the nose are lost and these larger particles tend to deposit in the TB region with high efficiency. An important characteristic of the TB region in the Task Group model is that this region is both ciliated and equipped with mucous secreting elements so that clearance of deposited particles rapidly occurs by muco-ciliary action to the throat for swallowing. Again, relatively soluble material may enter the systemic circulation.

The rate of mucous movement is slowest in the finer airways and increases toward the trachea. Since particles depositing in the tracheobronchial tree are probably distributed differently with respect to size, with smaller particles tending to deposit deeper in the lung, one expects larger particles to clear more quickly. Clearance of material in the TB compartment cannot be described by a single rate. TB clearance half-times from experimental studies imply that the larger airways, intermediate airways and finer airways clear with halftimes of about 0.5 hours, 2.5 hours and 5 hours, respectively (Morrow, et al., 1967; Morrow, 1972). It is relatively certain that material with slow dissolution rates in the TB compartment will not persist for longer than about 24 hours in healthy humans. The detailed nature of the mucociliary clearance mechanism has been recently reviewed by Schlesinger (1973).

The third compartment, the pulmonary or parenchymal region (P) represents the functional gas exchange sites of the lung. It includes respiratory bronchioles, alveolar ducts, alveolar sacs, atria, and alveoli. For particles to reach and deposit in this region they must penetrate the NP and TB regions on inspiration and either by settling or diffusion come into contact with pulmonary surfaces. Since a portion of each breath remains unexhaled, the times available for deposition may be long for some particles. Smaller particles are of primary importance in pulmonary deposition. Clearance from the pulmonary region is not completely understood, but the Task Group suggests several mechanisms including: (a) the dissolution of relatively soluble material with absorption into the systemic circulation, (b) direct passage of particles into the blood, (c) phagocytosis of particles by macrophages with translocation to the ciliated airways and, (d) transfer of particles to the lymphatic system including lymph nodes.

The fate of particles deposited in the P compartment is strongly dependent on the mechanical stability of the particles. Particles that undergo significant dissolution in the fluids found in the lung may dissolve while still within the air spaces, inside phagocytes or while in interstitial spaces. The Task Group (1966) recommended the use of three clearance half-times of 30 minutes, 90 days and 360 days for readily, intermediately and minimally soluble materials, respectively. An omitted factor in this clearance model is particle size (the rate of dissolution of a material in biological fluids being dependent on the available surface area of particles). At the present time clearance rates for the deep lung for humans are not known for many materials and the rates recommended by the Task Group provide a useful guide in the absence of detailed information.

The relative deposition of inhaled particles of various aerodynamic diameters as suggested by the Task Group for a moderate level of respiratory effort is shown in Figure 2. The total deposition and the fractional deposition in individual compartments are shown. The minimum total deposition at about $0.5 \mu\text{m}$ occurs since particles of this size are not strongly influenced by either inertial or diffusional forces.

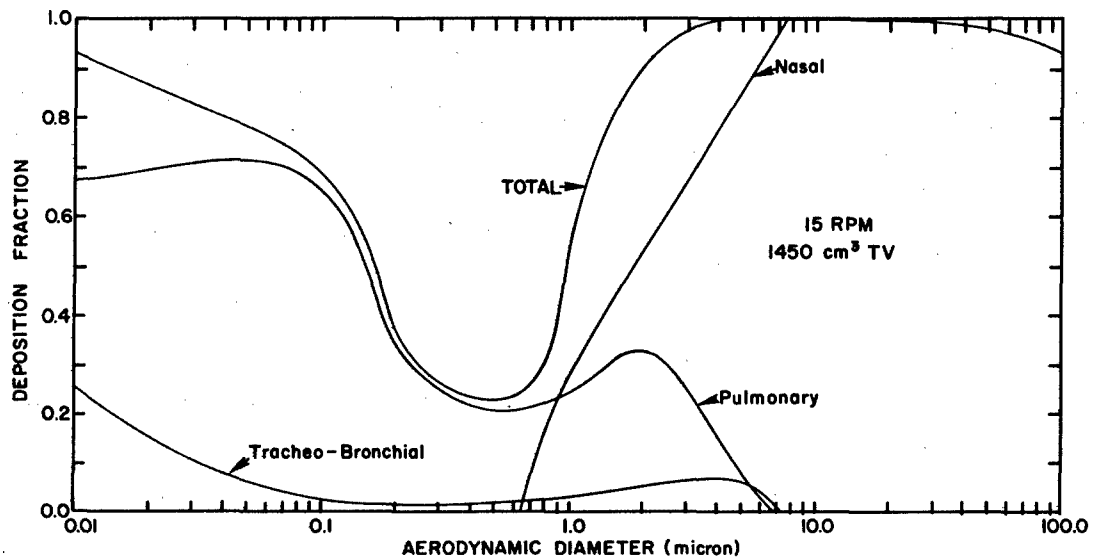


Figure 2. The deposition fraction of inhaled aerosols of various individual particle sizes with respect to aerodynamic diameter (μm) in the modeled regions of the human respiratory tract (assuming a respiratory rate of 15 per minute and a tidal volume of 1450 cm^3) as recommended by the Task Group on Lung Dynamics of the ICRP (1966).

TOXICITY AND PARTICLE SIZE

Particle size influences the toxicity of inhaled aerosols for a variety of reasons: (a) particle size affects the mass per particle and might therefore be expected to affect potential for hazard; (b) as described, the site of deposition within the respiratory tract as well as the clearance pattern is, to a great extent, influenced by aerodynamic size; (c) smaller particles have larger surface-to-mass ratios and therefore are more active with respect to chemical or physical interaction and rate of dissolution; (d) rate of phagocytic uptake may vary with particle size; and (e) particle size may influence the penetration of particles through membranes of the lung.

(a) Particle Mass

The total mass of material deposited in the respiratory tract is usually important in determining the potential toxicity for an inhaled aerosol. Hence, the deposition of a few particles that have a large mass

per particle may be more important than the deposition of numerous particles that are each small in mass. This fact is particularly relevant because the mass per particle for aerosols in the respirable size range can vary over many orders of magnitude. For example, since the mass of a spherical particle is proportional to the cube of the geometric diameter, 1000 particles of 0.1 μm diameter must be deposited in the lung to equal the mass burden from the deposition of but a single 1 μm diameter particle.

When particles of different individual masses are deposited in the respiratory tract, the number of cells which each directly affects may vary significantly with respect to mass per particle (depending upon the mechanism and range of influence). A given amount of mass deposited in the respiratory tract may be distributed among numerous small particles or among fewer large particles, and the effect on overall toxicity of these different situations may not be readily apparent. These considerations are probably less important for rapidly dissolved material that is in the particulate state only a brief time, and most important for material that is resistant to dissolution in the lung.

The case of relatively insoluble radioactive particles of alpha emitting materials deposited in the pulmonary region provides a timely example. Since the major direct effect of the particles on the surrounding cells relates to the alpha emissions, each aerosol particle irradiates a small surrounding volume of the lung. It can be argued that a given mass burden in the lung distributed among a few massive particles is less carcinogenic because the number of cells at risk is limited and those that are irradiated may in fact be over-irradiated and in effect sterilized, preventing development of neoplasia. On the other hand, it can also be argued that the distribution of the lung burden in larger particles is more hazardous because of the large local radiation doses received by cells surrounding the particles, and it is less hazardous to have smaller radiation doses which are associated with smaller particles (even though more cells are irradiated). This so-called "hot particle" question bears on the environmental impact of a nuclear technology and is currently being studied by many investigators.

(b) Aerodynamic Properties

Types of solid particles that can be identified with respect to their shape and concomitant aerodynamic character include: relatively globular particles that tend to approximate spherical shapes; plate-like or flat particles; long, thin particles or fibers; and clusters or agglomerates of particles. For relatively spherical particles of a given aerodynamic diameter, higher density particles have lower total mass. Hollow, or spongy particles of a homogeneous material will therefore have more mass per particle for a given aerodynamic size. Differences in toxicity with respect to particle density have not been demonstrated.

Long, thin fibers have aerodynamic diameters nearly independent of their length up to a length-to-diameter ratio of about 20 (Timbrell, 1972; Mercer, 1973). For this reason asbestos fibers containing considerable mass can behave like smaller particles aerodynamically and penetrate deeply into the lung. This effect is emphasized by the many cases of pulmonary asbestosis from the asbestos industry.

A particularly interesting aerosol in industrial toxicology, the metal fume, consists of chain-like agglomerates of particles smaller than $0.1 \mu\text{m}$ (Figure 3). Extreme toxicity is known to be associated with the inhalation of metal fumes. This may be due to the relatively large surface area associated with a given mass of fume aerosol. The aerodynamic drag on the large surfaces of fume particles allows them to follow airstreams and escape impaction in the NP and TB compartments. As in the case of the asbestos fibers, the ability of metal fume particles to penetrate to the deep lung undoubtedly contributes to their hazard.

(c) Surface Area

Two categories of toxic particulate materials can be identified with respect to mechanism of toxicity. Materials such as asbestos and quartz that are hazardous as solid particles, appear to have toxic shape or surface characteristics. Pneumoconioses in general are caused by the presence of intact particles. Other materials like Pb and Mn probably require dissolution, or at least some form of transformation from the original particle, in order to be toxic (Hatch and Gross, 1964). For both categories of particulate materials the specific surface, or surface-to-mass ratio, affects their toxicity. The surface-to-mass ratio for smooth spherical particles is equal to $6/\rho D$, where ρ is the physical density and D the geometric diameter. One micrometer diameter unit density particles have a specific surface of $6 \text{ m}^2/\text{g}$, while $0.01 \mu\text{m}$ particles have an area of $600 \text{ m}^2/\text{g}$. The increased toxicity of finely divided silica, discussed by Hatch and Gross (1964), appears to relate to increased surface area. The mechanism for toxicity appears to involve a tissue reaction to the particle surface.

A model for dissolution of particles in the P compartment that correlates well with experimental data on deep lung clearance has been proposed by Mercer (1967). The model assumes a rate of dissolution that is proportional to the available surface area of the particles. For materials that are toxic when dissolved, increased surface area tends to enhance toxicity. The dissolution of silver particles (mass median diameter = $0.04 \mu\text{m}$) in various aqueous media indicates that even a so-called "insoluble" material can undergo rapid dissolution when in a finely divided state (Figure 4).

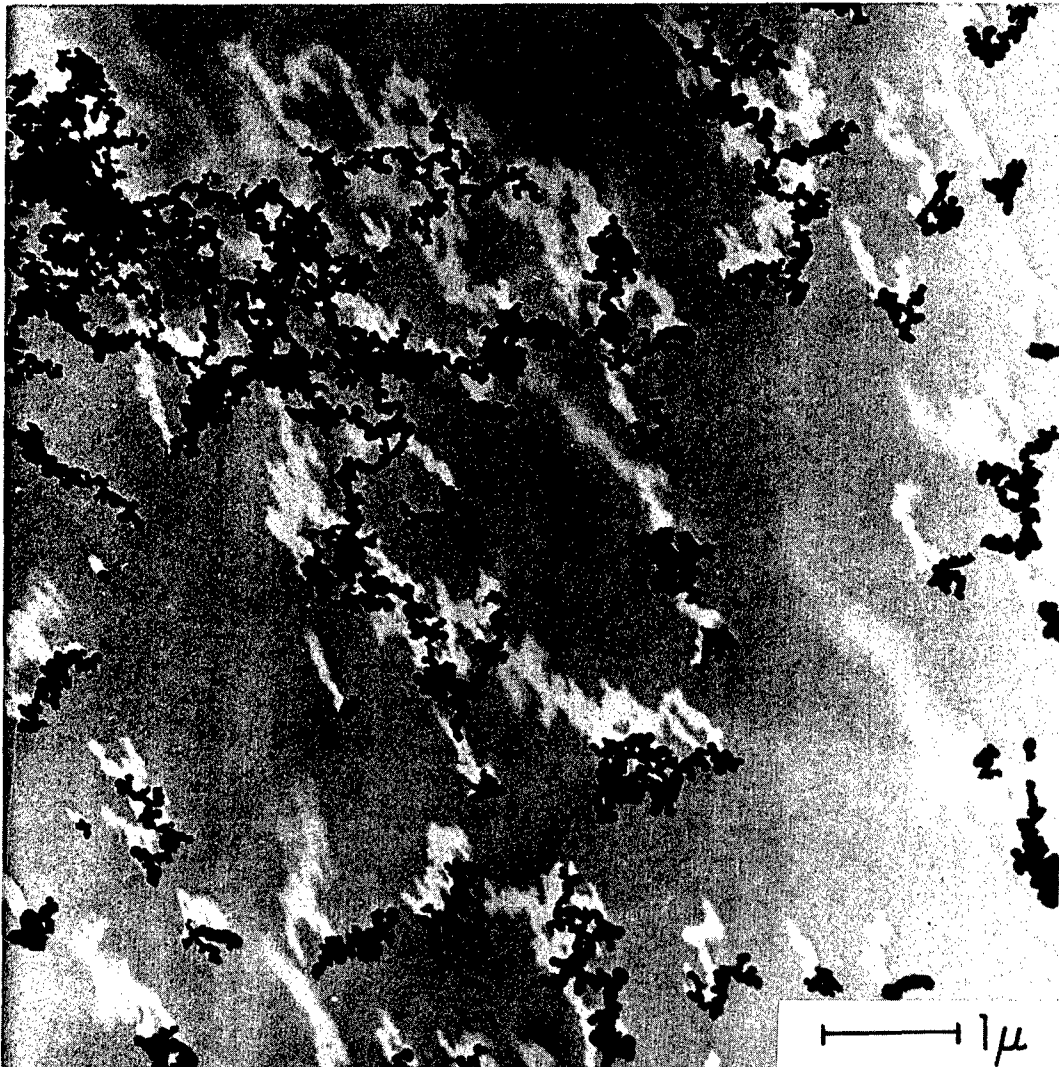


Figure 3. Electron micrograph of a sample of a metal-fume aerosol. The small, spherical primary particles, silver in this case, cluster to form branched chain-like agglomerates. Such agglomerates have large surface-to-mass ratios, can remain suspended in air for long periods (due to viscous drag) and can undergo rapid dissolution in the body. From Phalen (1972).

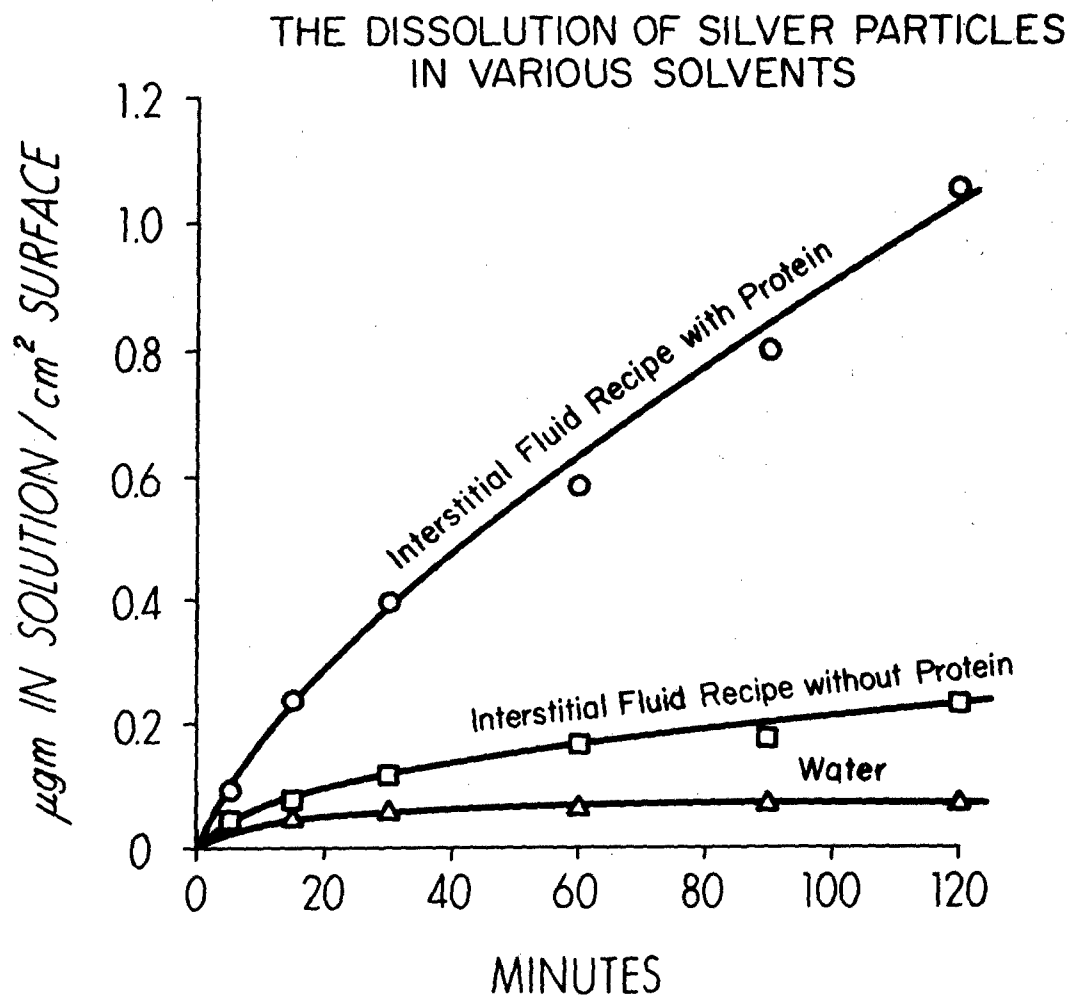


Figure 4. Dissolution of metallic silver particles (count median diameter = $0.03 \mu\text{m}$) in various aqueous media given as μgm dissolved per cm^2 of particle surface for various times up to 120 minutes. Gamble's interstitial fluid recipe with and without protein (bovine albumin) and distilled water were used. The increased rate of dissolution with protein present is probably due to the binding of silver ions to proteins. The silver particles used in this study are shown in Figure 3. From Phalen (1972).

On the basis of the dissolution rate found in the protein containing fluid, Mercer's model predicts that these silver particles should essentially completely dissolve in the lung in about 48 hours (Phalen, 1972). Systemic toxicants can be expected to be more rapidly dissolved and hence more hazardous when deposited in the lung as small particles.

(d) Other Size Dependent Factors

Aside from the influence of particle size on magnitude and distribution of dose, deposition pattern and dissolution rate, there are other size-related factors that may bear on toxicity. An optimal particle size of $1.5 \mu\text{m}$ for efficient uptake of polystyrene spheres by macrophages was suggested by Holma (1967). Holma (1967) gave an upper limit of $8 \mu\text{m}$ diameter for phagocytic uptake. The question of relative efficiency of uptake by macrophages of the lung for particles in the respirable size range (about 0.01 to $10 \mu\text{m}$) is worthy of further investigation.

The permeability of alveolar membranes to bare particles has been reported by Gross and Estrick (1954) and more recently by Tucker et al. (1973). In the earlier study rats were given small carbon particles ($\leq 0.2 \mu\text{m}$) by intratracheal injection. Nineteen hours later the particles were found extracellularly in interstitial spaces; considered by the authors to be proof of membrane penetration by bare particles. In Tucker's experiments carmine particles ranging from about $5 \mu\text{m}$ down to below $0.05 \mu\text{m}$ in diameter were inhaled by rats. At 3 hours post inhalation, microscopic examination revealed "small aggregates, up to cell size" in the extracellular interstitial spaces. Particulate material in the interstitium would presumably either remain, dissolve, undergo transport to lymphatic or blood vessels or return to the respiratory airway. The presence of interstitial foreign material for prolonged periods may lead to lung diseases. The role of particle size in membrane penetration is not yet well understood.

SUMMARY

The particle size distribution of inhaled aerosols is a factor in pulmonary toxicity for several reasons. Among those discussed are the relationship between particle size and amount of toxic agent per particle, the influence of aerodynamic and real size on the regional deposition within various anatomical regions of the respiratory tract and the effect of both deposition site and particle size per se on clearance kinetics. The role of particle size in the assessment of environmental hazards is one that is increasingly being realized as important.

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PULMONARY CHANGES INDUCED BY AMBIENT LEVELS OF OZONE.
A MORPHOLOGICAL STUDY.

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INTRODUCTION

The effects of ozone (O_3), a strongly oxidant gas, on the respiratory system have been studied by numerous investigators in recent years (Boatman et al., 1974; Castleman et al., 1973a; Castleman et al., 1973b; Chow et al., 1974; Freeman et al., 1974; Penha and Werthamer, 1974; Plopper et al., 1973a; Plopper et al., 1973b; Stokinger, 1965; Stokinger and Scheel, 1962; Stokinger et al., 1957; Stephens et al., 1974a; Stephens et al., 1974b). Much of the earlier work was confined to edemagenic concentrations of 1.0 ppm and higher (Scheel et al., 1959; Stokinger, 1965). Our interests have focused on the effects of lower levels, in the range of 0.2 to 0.8 ppm, which are found during moderate to severe episodes of photochemical smog in urban environments.

Previous light microscopic studies have indicated that the severest lesions of ozone are in centriacinar locations (Plopper et al., 1973a) and at lower concentrations damage is principally of epithelial lung components. Plopper et al. (1973a) in a histologic and ultrastructural study using rats exposed to 3 ppm O_3 for 4 hours and selective embedding processes confirmed this centriacinar location of the lesion. Recently, Stephens et al. (1974b) reported on the early response (6-48 hours) of the lung to ozone concentrations of 0.5 and 0.8 ppm. Their study combined surface changes as observed by SEM with histologic and ultrastructural observations.

Our objectives in this study were to define the lesions of ambient levels of O_3 (0.2, 0.5, and 0.8 ppm) after seven days of intermittent exposure using light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). SEM provided the advantages of observing large tissue samples and correlation of surface alterations with histologic and ultrastructural changes. This study was part of a larger one designed to correlate

biochemical and morphological findings following O₃ exposure and to compare the effects of continuous and intermittent daily exposure regimens. A portion of the biochemical results and the histochemical results have been published previously (Castleman et al., 1973a; Castleman et al., 1973b; Chow et al., 1974).

MATERIALS AND METHODS

Male Sprague-Dawley rats 70 ± 3.5 days of age were used; they were from a colony free of chronic respiratory disease and were housed under microbiological filters for one week prior to use. Twenty rats were divided into four groups which furnished animals for three exposure levels and one control group.

The levels of O₃ selected were 0.2, 0.5, and 0.8 ppm and an intermittent regimen of exposure was chosen which consisted of 8 hours of exposure during late evening hours to coincide with peak activity periods of the rats during darkness. For exposure, rats were housed in pairs in wire mesh cages in a 21 cubic foot fiberglass chamber (Germfree Labs., Inc., Miami, Florida). Ozone was generated from bottled pure oxygen by a silent electrical discharge ozone generator and mixed with CBR filtered room air. Ozone concentration within the chambers was continuously monitored (including the control chamber) with a Mast meter which was calibrated and intermittently checked using a neutral-buffered 2% potassium iodide method (Saltzman, 1965).

At the end of the seventh exposure period, rats were removed from the chambers and anesthetized with sodium pentobarbital. A thoracotomy was performed and the thoracic viscera was immediately removed. The trachea was cannulated and the lungs fixed by airway perfusion with Karnovsky's fixative (Karnovsky, 1965) at 30 cm of water pressure. Following a short period of storage the right diaphragmatic lobe was sectioned with razor blades and adjacent areas selected for LM, SEM, and TEM.

Light Microscopy

Sections were routinely embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Paraffin sections were used to screen rats for absence of lesions of spontaneous respiratory diseases and to evaluate uniformity of perfusion and fixation. Three rats from each group were selected for ultrastructural study. Additional light microscopic study was done on one micron plastic embedded tissue as discussed below.

Scanning Electron Microscopy

Lung samples, no larger than 1 cm square, which contained a longitudinal section of conducting airway and one or more terminal bronchioles were selected for SEM. Tissues were dehydrated in a graded series of alcohol. Absolute alcohol was substituted with amyl acetate which was then exchanged for liquid CO₂ in a critical point drying apparatus (Denton) (Anderson, 1951). Following passage through critical point the dried tissues were coated with silver and gold or carbon and gold palladium in a high vacuum evaporator (Denton). Coated tissues were stored in a desiccator until examined on an ETEC autoscan SEM.

Transmission Electron Microscopy

Tissue specimens, where practical, were complementary halves of SEM specimens. Tissue for TEM was prepared using the large epoxy-embedded block techniques of Lowrie and Tyler (1973). Sections one to two microns thick were cut, mounted on glass slides and stained with Richardson's trichrome stain. Specific areas were selected from these blocks and, using the mesa technique (Lowrie and Tyler, 1973), sectioned for TEM followed by routine staining with uranyl acetate and lead citrate. Examination was completed on a Zeiss 10 EM.

RESULTS

The normal light and ultrastructural morphology of the rat lung has been discussed by several investigators and our findings in control rats are in agreement with previous descriptions (Plopper et al., 1973a; Stephens et al., 1974b).

Light Microscopy

One micron sections of plastic embedded tissue were most useful for critical histologic evaluation of the lungs. The lesions caused by ozone were most evident in the distal portion of the terminal bronchiole and proximal alveoli of the alveolar duct. Features of the lesions were similar for each level of exposure, but the degree of severity and extent of the lesion increased with increased level of exposure. Lesions produced by any one level could be distinguished from those caused by the other levels, and each in turn could be distinguished from the control group. Within any one lung, however, there were various degrees of involvement of centriacinar regions.

The surface of terminal bronchioles from 0.8 ppm exposed rats was coated with a minimal amount of eosinophilic granular debris which infrequently entrapped solitary vacuolated macrophages. Alveoli in proximal portions of alveolar ducts were paved with clusters of macrophages and sporadic neutrophils. The macrophages were often prominently vacuolated. Single small mononuclear cells resembling lymphocytes were infrequently observed associated with clusters of macrophages. Neutrophil numbers varied considerably among rats and from one region of the lesion to another in an individual rat. They were generally most prevalent in proximal alveoli and adjacent interstitium. The cytoplasm of alveolar epithelium appeared more prominent (thickened) and nuclei were more frequent in proximal alveoli. Interstitial tissues of interalveolar septa were thickened by accumulations of mononuclear cells, infrequent neutrophils and eosinophilic hyaline material. Cells and supporting substance of the interalveolar septa were loosely arranged suggesting separation by edema fluid of low protein content (Figure 1A).

Lesions at the 0.2 ppm exposure level were mild in comparison to the 0.8 ppm group. Changes were confined to proximal alveoli of the alveolar duct and adjacent interalveolar septa. They were characterized principally by accumulations of macrophages in alveolar lumens and an occasional mononuclear cell in interalveolar septa. It was unusual to find more than 2-3 macrophages in an alveolus and this occurred only in the first 2-3 alveoli of a pulmonary acinus. Infiltrating cells other than macrophages were uncommon (Figure 1B).

Lesions observed in the 0.5 ppm exposure group were similar in character to those described above and of intermediate magnitude.

Changes observed in the trachea and subsegmental bronchi were limited to the two highest exposure levels and consisted of increased granular debris on the epithelial surface and occasional pyknotic cells intermixed with debris (Figure 2).

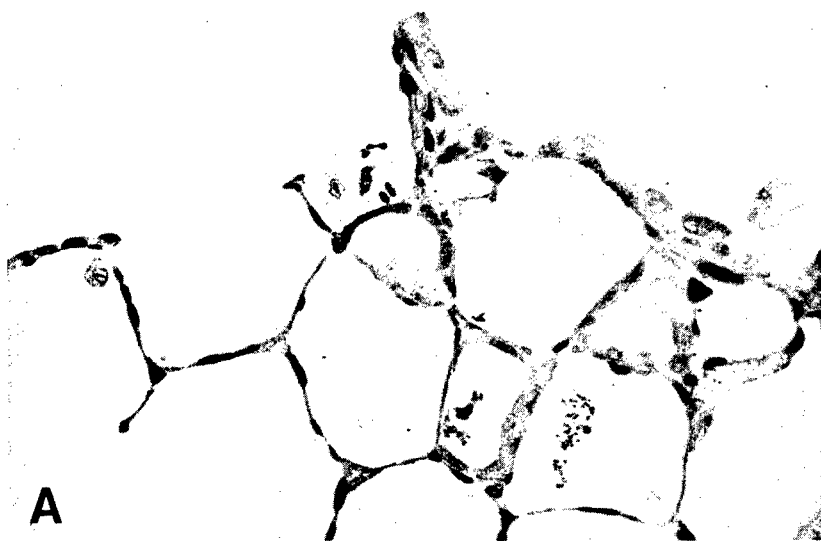


Figure 1.

A. The extent of lesions in proximal alveoli of 0.2 ppm ozone was difficult to appreciate by light microscopy. Minimal numbers of alveolar macrophages were identified. Compare to Figure 3A.

B. A similar area from an 0.8 ppm exposure demonstrates the abundance of macrophages in proximal alveoli as well as the prominent thickenings of alveoli septa.

A. x 400.

B. x 400.

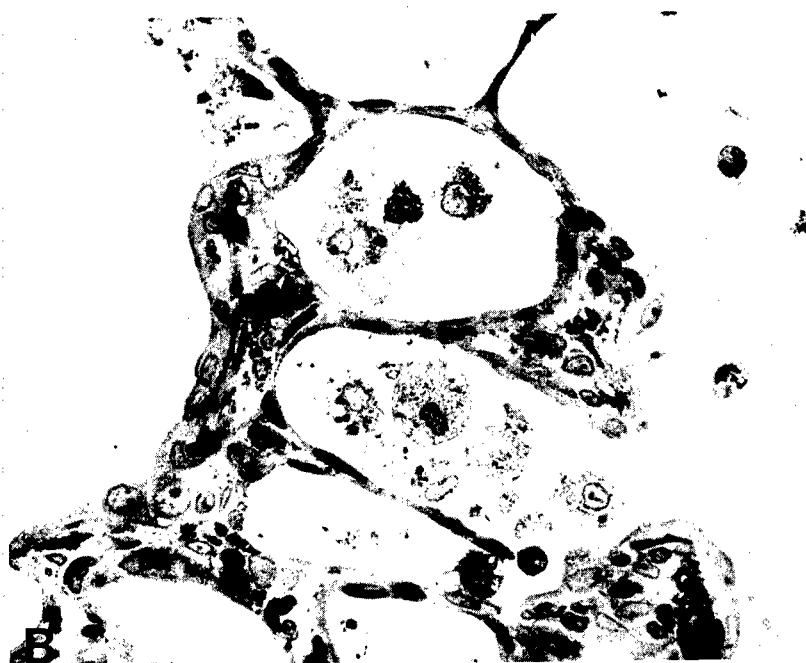




Figure 2. Pyknotic sloughing ciliated cells were observed in larger airways. Evidence of damage to nonciliated cells at this level was not observed. x 2,120.

Scanning Electron Microscopy

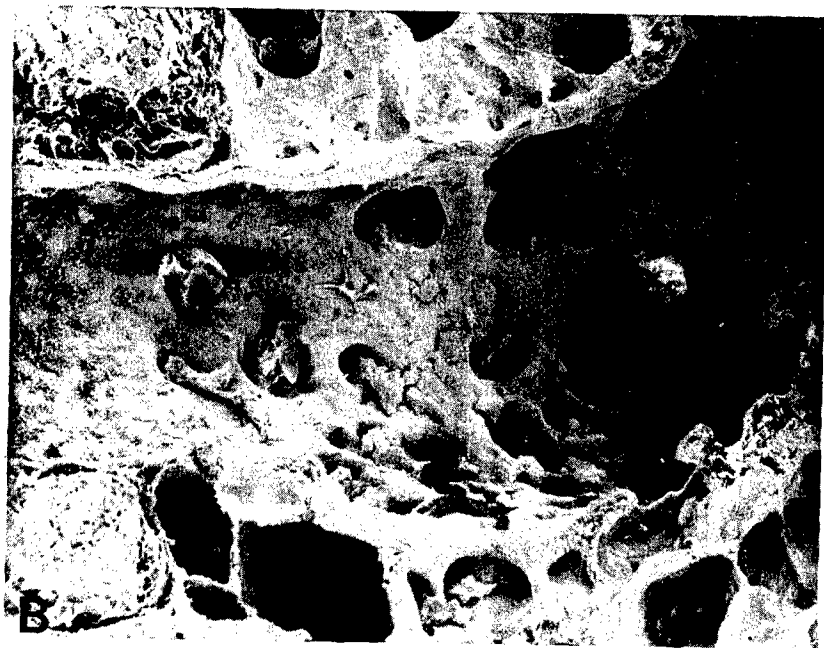
Of the three forms of microscopic evaluation employed, SEM afforded the quickest and most effective means to survey a large tissue sample and to identify areas damaged by ozone. This was especially true at the 0.2 ppm level. The large depth of field revealed a large surface area of terminal bronchiole and adjacent structures for evaluation and provided the most sensitive morphological indicator of surface damage and cellular accumulation.

Surface changes were most dramatic in centriacinar regions, which corresponds with the histologic observations. Proximal alveoli of alveolar ducts were paved and occasionally occluded by clusters of infiltrating inflammatory cells having a roughened granular surface and frequent filamentous pseudopodia (Figure 3). These filamentous extensions overlaid and intertwined with similar adjacent cells and extended to the alveolar surface. Occasional cells having a more flattened appearance and blunt pseudopodia



Figure 3.

A. Alveolar macrophage accumulation in proximal alveoli served as indicators of ozone damage in 0.2 ppm exposed animals.



B. Massive macrophage accumulations were easily identified in proximal alveoli of 0.8 ppm exposed animals. Distal alveoli of adjacent pulmonary acini (top center of photo) were free of inflammatory cell infiltration.

A. x 236.

B. x 330.

were applied to alveolar walls. At the lowest exposure level, these cell accumulations were confined to the first few proximal alveoli of alveolar ducts; however, with increased ozone levels inflammatory cell accumulations extended more proximally and distally but still never reached to the most distal alveoli of alveolar ducts. The surface of epithelial cells in proximal alveoli of alveolar ducts in exposed rats had an increased number of microvilli which produced a roughened granular appearance.

Alterations in distal portions of terminal bronchioles were easily recognized. Clara cells of control rats had a prominent centrally-located cytoplasmic projection which protruded into the bronchiolar lumen as high as, or higher than, cilia of adjacent ciliated cells. The surface of this projection was relatively smooth while the more basilar portions and peripheral borders of the cell had a blunt microvillar surface. Clara cells in 0.8 ppm ozone exposed rats were completely flattened. Their luminal surfaces were composed of numerous small cytoplasmic blebs and microvilli (Figure 4).

The surface of a normal terminal bronchiole had a reticulated appearance produced by the pattern of distribution of ciliated cells and Clara cells. The cilia bordering Clara cells tended to be of uniform length, diameter, and density. In exposed rats there was a paucity of cilia and those present varied considerably in length and many of the longer cilia were atypical having focal blebs and blunted ends. Damage to cilia was most obvious in the rats exposed to 0.8 ppm and was severest at the most distal end of the terminal bronchiole.

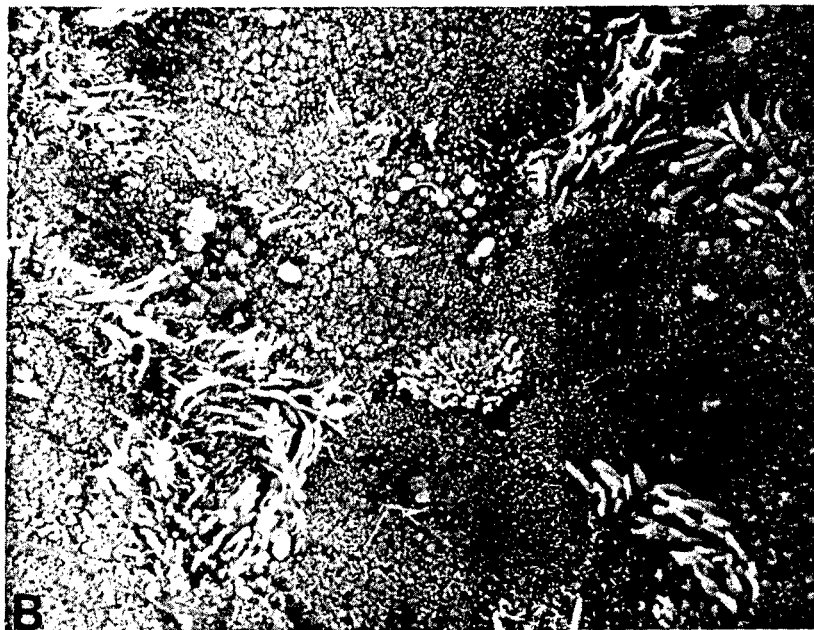
Changes in the terminal bronchiole were present at the 0.2 ppm exposure level but were not as pronounced. The cytoplasmic projections of Clara cells were present but were of limited height in the most distal portion of the bronchioles. Clara cells of the proximal terminal bronchiole appeared normal. Cilia still appeared less uniform than in control animals and were not as dense.

In subsegmental bronchi, ciliated cells appeared to be the only indicator of damage. Randomly distributed clusters of cells sparsely populated with cilia were observed. Infrequently, tightly coiled masses of cilia were observed lying on top of airway epithelium. These were interpreted as pyknotic sloughed ciliated cells. Damage to ciliated cells was also observed in the trachea where focal areas of reduced density of cilia were recognized and those present were of variable diameter and length. Evaluation of the trachea required identification of specific areas since epithelium overlying cartilagenous rings tended to be heavily ciliated and sparsely populated with nonciliated secretory cells whereas epithelium of the intercartilagenous area was essentially the opposite in that it had predominately nonciliated epithelium. Damage to cilia in subsegmental bronchi and in the trachea was not observed in rats of the 0.2 ppm exposure level.



Figure 4.

The surface appearance of terminal airways from control animals (A) can be compared to a similar area from an 0.8 ppm exposed animal (B). Flattening of Clara cells and increased granularity of their surface was observed as well as damage to adjacent cells.



A. x 2,520.

B. x 3,652.

Transmission Electron Microscopy

Corresponding with the LM and SEM observations, the brunt of the damage occurred in the centriacinar portion of the pulmonary acinus. The plastic embedment of large tissue samples provided the necessary identification of specific sites to be used for TEM. Cells infiltrating the proximal alveoli of alveolar ducts were predominately macrophages which contained an abundance of secondary lysosomes. Most macrophages were highly vacuolated, had numerous cytoplasmic projections, and frequently contained remnants of osmiophilic lamellar bodies. A few small mononuclear cells resembling lymphocytes were observed either in close contact with macrophage clusters or as isolated individual cells. Minimal numbers of polymorphonuclear cells were observed with the clusters of macrophages.

Alveolar epithelium was observed in various phases of degeneration and modification. Membranous pneumonocytes (Type 1 alveolar epithelium) were observed in different stages of swelling and fragmentation. This Type 1 cell damage was confined to proximal alveoli ducts and when observed appeared to extend over the entire portion of the cells visible in the section. The swollen cells remained attached to the basement membrane and projected into the alveolar lumen. Portions of the alveolar epithelium consisted of thickened elongated cells similar to Type 1 alveolar epithelium but containing features of granular pneumonocytes (Type 2 alveolar epithelium) such as microvillar surface and cytoplasmic osmiophilic lamellar inclusion bodies. Type 2 pneumonocytes were frequently observed in pairs or clusters, suggesting that they were proliferating, but mitotic figures were not observed (Figure 5).

The infiltrating cells observed by LM in the interalveolar septa were difficult to identify specifically as to cell types even by TEM. A majority of cells within interalveolar septa were mononuclear cells having a moderate to abundant amount of cytoplasm containing many polyribosomes and a few mitochondria. This cell was rounded or occasionally spindle-shaped and was never observed passing into alveolar lumens. Collagen strands were easily identified in septa but rarely observed originating from the mononuclear cells. Cells of the interstitium were loosely arranged and separated by clear spaces suggesting mild interstitial edema. Septal thickening appeared to result primarily from separation of existing components by clear vacuolated areas and it was difficult to appreciate an increase in amount of collagen within septa. Endothelial cells did not appear altered. Neutrophils were observed infrequently in interalveolar septa.

At the 0.2 ppm level of exposure, alveolar macrophage accumulations were still observed in proximal alveoli, generally as single cells and seldom in clusters. Swelling of Type 1 epithelium was not observed and areas of proximal alveoli paved with modified thickened Type 2 epithelial cells were not observed. Mononuclear cells were found in interalveolar septa in limited amounts.

Lesions of the terminal bronchiole were characterized by changes in both ciliated and nonciliated (Clara) cells. The cytoplasm of numerous ciliated cells contained clusters of basal bodies and precursors of basal bodies suggesting the formation of new cilia. Cilia were observed to be irregular in shape having focal cytoplasmic swellings. The cytoplasmic projection of Clara cells which normally contains smooth endoplasmic reticulum was seldom observed in distal terminal bronchioles of exposed rats.

Ciliated cells of subsegmental bronchi and trachea also contained precursors of basal bodies and clusters of formed basal bodies in apical portions of their cytoplasm. Occasional pyknotic ciliated cells were observed on the surface of these airways. Mucous-secreting cells of exposed rats especially at the higher levels were observed with droplets being exocytosed from the apical cell surface. Homogeneous droplets resembling those within cells were seen entrapped among the cilia of rats exposed to the upper two ozone levels. Changes suggesting damage to organelles of airway lining epithelium were not observed. Mitotic figures were observed along the basilar portions of the trachea and subsegmental bronchi suggesting increased turnover of airway epithelium in 0.8 and 0.5 ppm exposed animals.

DISCUSSION

This study confirmed that intermittent daily exposure of rats to levels of ozone as low as 0.2 ppm induces predominantly centriacinar pulmonary lesions. This focal induction of changes made morphologic evaluation and interpretation difficult and required precise identification of areas of lung and pulmonary acini being examined. At all levels of morphologic evaluation, it appeared that infiltration of alveolar macrophages into proximal alveoli of alveolar ducts was the most sensitive indicator of damage. Since SEM enables evaluation of a large surface area of a pulmonary acinus rapidly and with proper identification of areas being examined, it provided the quickest and simplest, sensitive means to search for ozone damage. In addition, changes observed by SEM were often difficult to appreciate by LM or TEM and further served to demonstrate the value of a combined SEM, LM, and TEM morphological approach.

Figure 5. Proximal alveoli contain alveolar macrophages, many with fragments of lamellar osmiophilic inclusions in secondary lysosomes. A microvillar surface was observed on an increased number of cells lining alveoli plus a wider cytoplasmic lining and occasional lamellar inclusion all features of granular pneumonocytes. x 5,152.



At the gas exchange level, Type 1 epithelium appeared to be very sensitive to ozone damage as demonstrated by the swelling and fragmenting of these cells. This observation is in support of work by Plopper et al. and their observations of swelling and necrosis of membranous pneumonocytes and the apparent resistance of granular pneumonocytes to 3 ppm O_3 for 4 hours (Plopper et al., 1973a). Stephens et al. (1974a; 1974b) have also reported that membranous pneumonocytes are very sensitive to 0.5 and 0.8 ppm O_3 . Even after an exposure period as short as two hours, this cell type was severely damaged or removed from large areas of basement membrane. The clusters of Type 2 cells suggests that this cell type is proliferating focally, perhaps attempting to replace damaged Type 1 cells. The replacement of Type 1's with Type 2's is a premise held by several and remains to be investigated further. Evans et al. (1973) suggested that in rats following nitrogen dioxide exposure the mechanism for alveolar epithelial cell renewal was that Type 2 cells were the progenitor cells for Type 1 cells. Work by Adamson and Bowden in mice and fetal rats has supported this hypothesis of cell renewal (Adamson and Bowden, 1974a and 1974b).

The factors which are responsible for the accumulation of macrophages at this centriacinar site are unidentified. It was of interest that lymphocytes were present in limited numbers among the infiltrating inflammatory cells. Their association with the production of the lesion or in its prolongation is unclear. Stokinger and Scheel (1962) and Scheel et al. (1959), however, have previously speculated on the involvement of the immune system in ozone toxicity and have demonstrated the production of antibodies against ozone altered animal proteins. Further investigation is needed to define the influence humoral and cellular immune functions have on the pathogenesis of this lesion.

The macrophages within the lesion contained an abundance of osmophilic lamellar inclusions and although the source of this material was uncertain, it is possible that a massive release of osmophilic inclusions from granular pneumonocytes occurred during exposure as a protective mechanism. Plopper et al. (1973b) have critically examined the ultrastructural features of pulmonary alveolar macrophages following acute ozone exposure. Their conclusion was that ultrastructurally detectable damage to these cells was not produced; however, two morphologically distinguishable populations of macrophages were observed: those which were similar to macrophages in unexposed lungs and those containing an abundance of large granular cytoplasmic inclusions, undoubtedly the result of extensive phagocytic activity. Our findings concur with their conclusions.

Cells accumulating in the interstitium, termed unclassified mononuclear cells, may represent one of several cell types: fibroblasts, lymphocytes and/or blood monocytes destined to become alveolar macrophages. It is felt that a majority represent the latter possibility since macrophages are

rapidly accumulating in the lesion and present evidence supports bone marrow and blood borne monocytes as the source of alveolar macrophages (Bowden, 1972; Velo and Spector, 1972). A minimal number of these cells may become fibroblasts but following a seven day exposure few can be observed in close association with collagen bundles or interstitial fibrillar material to suggest production of the same.

Ciliated cells of the terminal bronchiole and upper airway appear to be the only indication of damage in this location; however, damage to these cells is a less sensitive morphological indicator of ozone damage than is the lesion of the proximal acinus. The clusters of basal bodies and precursors of basal bodies in ciliated cells represent evidence of regeneration of cilia (Sorokin, 1968). The variation in density and length of cilia as observed by SEM would seem to support the idea that cilia are in different phases of damage and replacement.

This study has demonstrated the usefulness of a three-fold approach to morphological characterization of a pulmonary lesion. It has served to support existing evidence that ozone at ambient levels induces principally a centriacinar lesion involving the crucial point where conducting airways transform to gas exchange regions. The lesion as a bronchiolitis of the smallest conducting airway has the potential of progressing to an obstructive lesion. Changes of a less severe nature have been described in large conducting airways which affect primarily ciliated epithelium. Finally it has been demonstrated that levels of 0.2 ppm ozone can induce centriacinar changes in the rat lung and that additional work is needed to define the lowest threshold limit.

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THE USE OF CELL BIOLOGY IN ENVIRONMENTAL TOXICOLOGY

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My assignment is to discuss the application of cell biology to Environmental Toxicology using any time period up to 20 minutes for this purpose. This may turn out to be a disaster. Not being an environmental toxicologist (nor even a toxic environmentalist) puts me at a substantial disadvantage. Devoting a maximum of 20 minutes to such a broad topic as cell biology is roughly equivalent to attempting to compress Genesis from 6 days to 1 day.

Be that as it may, perhaps a suitable starting point might be an attempt to characterize classical toxicology by analyzing the program which has been presented here during the past few days.

One characteristic is that most studies involve studies at an organ level or studies of intact animals. Typical studies are performed by exposing several animal species to possibly toxic agents for given periods and then evaluating survival time, morphologic changes and certain constituents* of the blood and urine. Another general characteristic is that a diffuse and basically isolated and independent series of observations have made up the bulk of the presentations. The individual pieces of work are undoubtedly excellent and each paper does add something to scientific reality, but how do the various pieces fit? And assuming continued studies based on seemingly unrelated problems, will a cohesive picture ever emerge? The challenge then is to attempt to provide a general approach for studying the effects of toxic agents on biological systems. Such an approach should hopefully provide cohesion as well as relevance and precision of interpretation.

One unifying approach is to consider that all effects of toxic agents are based on alterations of cellular function. Neural toxicity caused by methylbutylketone exposure must ultimately result from abnormalities of function in single neurons. Sterility in minks caused by chemical exposure must ultimately result

*Surprisingly, these studies do not seem to take advantage of currently available automated screening chemical techniques which provide measurements of many different chemical constituents in patients.

from abnormal function of reproductive cells in that species. Altered myocardial function related to halogenated hydrocarbons or Freon must ultimately be caused by altered function of single myocardial cells. Can a general model be developed which both provides a guide to studying altered cell function caused by individual toxic agents and also provides a unified approach to the study of most toxic agents?

This morning I would like to suggest the broad outlines of such a model and then indicate how this model might be coupled with the traditional methods of classical toxicology to provide new insight into mechanisms of the effects of toxicants on biologic systems.

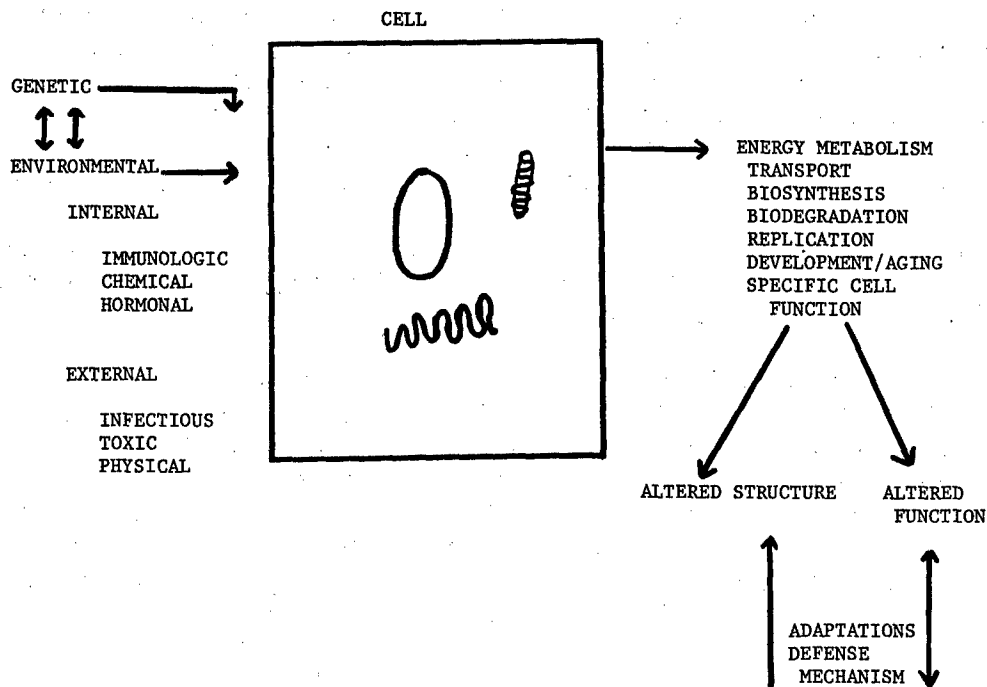


Figure 1. Model for analysis of cell injury.

Cell injury arises from either genetic alterations, environmental alterations or as indicated by the two headed arrows, a combination of the two since genetic factors may predispose to environmental injury and environmental factors can produce genetic abnormalities. Environmental abnormalities are of 2 types - those arising from the internal environment (immunologic reactions, endogenous chemical mediators and hormones) and those arising from the external environment (toxic agents, infectious agents and physical factors such

as temperature, ionizing radiation, pressure, etc.). In turn, environmental and genetic factors impact on various cell types altering one or a combination of basic cell functions. These functions include altered energy metabolism since all living cells are open ended systems requiring a continual supply of energy; altered transport since living cells require ongoing influxes of ions, water and substrates from the outside and the outflux of a wide variety of cellular products and wastes; altered biosynthesis and biodegradation since structural and functional elements of all cells are being continually destroyed and replaced; altered growth and development since all cells have cycles of developmental changes; alterations of aging since all cells have a finite life span (even in the absence of environmental or genetic injury) and alterations of specific cell functions (for example, pinocytosis and phagocytosis by macrophages; contraction by muscle cells; secretion by endocrine cells etc.). Alterations of one or a combination of these basic cell functions will produce alterations of cell structure which can be detected and quantitated by appropriate morphologic techniques and alterations in cell function which can be detected and quantitated by appropriate measurements of cell function.

As indicated in Figure 1, alterations in cell structure and function often evoke both adaptive changes and a variety of defense mechanisms which modify the primary alterations in basic cell function. Not uncommonly, these adaptive and defense responses while mitigating the effects of the primary disturbance produce additional structural and functional abnormalities.

Given this general model, how can such a model be coupled with classical toxicological studies?

- 1) CELL TYPES AFFECTED
- 2) DOSE - RESPONSE RELATION
- 3) TOXICO KINETICS
 - RATE OF UPTAKE
 - POOL SIZE
 - DISTRIBUTION
 - RATE OF EXCRETION
- 4) STRUCTURAL AND ULTRASTRUCTURAL ABNORMALITIES
- 5) MECHANISM(s) OF ACTION
 - ENERGY METABOLISM
 - BIOSYNTHESIS
 - BIODEGRADATION
 - TRANSPORT
 - REPLICATION
 - DEVELOPMENT AND AGING
 - SPECIFIC CELL FUNCTION
- 6) ADAPTATIONS EVOKED
- 7) PREVENTION AND REVERSAL

Figure 2. Application of cell pathophysiology to toxicology.

Figure 2 outlines a seven step process which provides for such an application. Whole animal studies are useful in identifying specific cell types (step 1) affected by a given toxic agent. Moreover, such studies help determine whether studies of altered cellular biology would optimally be conducted on whole animal preparations, on isolated cells prepared by various techniques for cell separation, or on cells maintained in tissue culture. Whatever biological system is adopted for study, dose-response relationships in intact animals (step 2) require definition both to design appropriate single cell studies as well as to determine the biological importance of the given agent. In whole body systems, the characterization of toxico-kinetics (step 3) of the agents being studied is critical both to determine the active blood or tissue levels of toxic agent and for optimal design of single cell studies. Toxico-kinetics, of course, is not only relevant to cell biology. The characterization of toxico-kinetics of potential toxic agents would appear to be of key importance in toxicological studies generally. A similar approach in pharmacology (pharmaco-kinetics) has produced major breakthroughs in the understanding of drug action. It is surprising that a similar approach has not been widely used in toxicology. A systematic evaluation of the rate of uptake, pool size, distribution and the rate of excretion should be part of the study of every specific toxic agent. For example, studies of maximal allowable concentrations of a given inhaled agent in, say, the mouse may have little relevance for man. These two species have such different rates of alveolar ventilation that extrapolation from mouse to man may not be warranted without knowledge of toxico-kinetics.

Given the first 3 steps, it becomes feasible to proceed to the study of cell function in depth. For some purposes, generally available cell types which have been well characterized biochemically and biophysically will be the most useful - cells such as HeLa tumor cells, macrophages, cultured fibroblasts, or red cells. A large amount of data relative to cell function has been accumulated for these cell types. These data can be used as background for the contemplated studies. For other studies, specific cell types should be used since available data will suggest injury to these specific cell types alone.

Obviously, structural and ultrastructural observations (step 4) will represent one important element in the analysis of cell injury and frequently will provide insight into the mechanisms of altered cell function. It should be recognized, however, that structural changes tend to be nonspecific, a wide variety of different abnormalities giving rise to the same structural alteration. Structural studies often lack acceptable resolution of time relationships in the development of abnormalities. Therefore, studies of structure without studies of function are always incomplete.

Step 5 involves studies of basic cell function. As a first approximation, screening measurements may be used to determine which basic cell functions might be altered by a given toxic agent. Changes in mitochondrial oxygen uptake and the rate of lactate generation can be used to screen the possibility of altered energy metabolism. Alterations of biosynthesis/biodegradation can be detected

by appropriate kinetic measurement of various chemical species. For example, the rate of incorporation of amino acids into the general protein pool and the rate of release of labelled amino acids from protein provide a basis for assessing biosynthetic and biodegradative processes involving protein metabolism. The possibility of general transport abnormalities can be defined by looking at Na^+ transport. Replication can be investigated by looking at DNA metabolism. Development and aging can be at least qualitatively described in tissue culture systems. Hosts of indices are available for examining specific cell function and relating abnormalities found back to the other basic disturbances. Given appropriate data derived from these screening studies of altered basic mechanism, it is theoretically possible to pursue each specific abnormality ultimately to the molecular mechanisms involved and therefore to examining adaptation (step 6) and prevention and treatment (step 7) on a rational basis.

Several general considerations should be emphasized. This discussion should not be construed as being "anti whole animal" studies. Cell physiology on one hand and organ physiology on the other represent two sides of the same coin. Both approaches are necessary. My emphasis reflects an impression that existing studies are somewhat unbalanced in using chiefly an organ or whole body approach. There is obviously nothing new or unique in the specific approach to cell biology which has been presented. It is easily possible to develop other specific approaches which are at least as useful as this one. The important element is that systematic studies of cell function should be an indispensable part of toxicologic investigations. What precise format should be used is arguable.

Aside from the intrinsic advantages that cell biology provides in clarifying biological mechanisms, there are two specific advantages in the area of environmental toxicology. Experimental exposures to toxic agents cannot be done in human subjects. It is therefore necessary to perform such studies on a variety of non-human species and extrapolate the results to man. In practice, numbers of mammalian species (rarely nonmammalian species) are studied. This practice requires the assumption that any changes found are similar in man and the other species. Such an assumption may be quite unwarranted. There are no data for example, that one day in the life of a mouse is equal to one day in the life of man. Indeed, since life span in the mouse averages about 2 years and in man averages 3 score and 10, there is substantial doubt that one day in each life is equivalent. What then should be one's view of experiments in which nonhuman species are exposed to a given toxic agent for 8 hours a day, 5 days a week to mimic human occupational exposures? And how rigorous are maximal allowable concentrations obtained from this type of experiment? The use of cells derived from humans for studies of altered cell function is, of course, entirely practical. This represents a specific advantage of the approach which has been outlined above.

Another specific advantage is related to the increasing rate at which toxic agents are being developed. Moreover, the dimensions of toxicology have been broadened to include exposure to unknown pollutants in the air, in water, in food,

etc. Thus, the demands for toxicologic testing are increasing. Whole animal studies by their very nature are logistically complex. Intensive screening of toxic agents in whole animals calls for major investments in time, money, and space. Isolated cell studies can presumably provide a more practical solution for mass screening than similar studies in whole animals. Since the precise relationship between changes in whole animals and cells may be complex, basic data would be required to determine to what extent studies of cell function could substitute for whole animal studies. But, obtaining such data would clearly be of major importance.

In summary, studies of altered cellular structure and altered cellular function produced by toxic agents is an important and probably neglected component of toxicology. It is possible to develop a rigorous and systematic approach for studying altered cell function produced by toxic agents. Aside from the practical advantages of such an approach, it offers the conceptual advantage of providing a unified interpretation of seemingly diverse phenomena as well as potentially leading to an understanding of molecular mechanisms of altered function. In turn, such understanding could lead to more rational approaches to prevention and treatment.

PULMONARY CELLULAR TOXICITY OF CADMIUM

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INTRODUCTION

There are numerous instances of accidental industrial exposure to cadmium fume which have resulted in severe respiratory damage (Kazantsis et al., 1963). Some of the survivors were later shown to have severe emphysema of the centrilobular type (Lane and Campbell, 1954) which suggested that animals given an appropriate exposure of cadmium by inhalation would provide a reproducible model of emphysema.

Multiple CdCl₂ Aerosol Exposure and Centrilobular Lesions

In the initial studies undertaken in our laboratory (Snider et al., 1973) male rats, 150-300 g. body weight, were exposed for one hour daily to an aerosol of 0.5% cadmium chloride (CdCl₂) in physiologic saline. The aerosol was generated by a standard deVilbiss 35A ultrasonic generator which gave particles with a mean size of 3.5 μ (range 0.5 μ to 8.0 μ) (? actual dosage/air flow).

Three groups of rats were exposed to the aerosol on 5, 10 and 15 occasions respectively and were killed at two hours, 3 days and 10 days after the last exposure. They were killed by barbiturate overdosage and the lungs were inflation fixed with formalin at a hydrostatic pressure of 25 cm. Two hours after five exposures, the animals showed acute pneumonitis centered about bronchioles which was replaced at three days by granulation tissue. Healing had occurred by 10 days leaving fine peribronchiolar fibrosis and minimal alveolar distortion. With 10- and 15-hour exposures to CdCl_2 , the acute inflammatory changes were less marked but the resulting peribronchial fibrosis and alveolar destruction was greater and gave rise to a focal lesion resembling that of the centrilobular lesion described by Leopold and Gough (1957). The progression of morphologic events suggested by these observations is summarized in Figure 1.

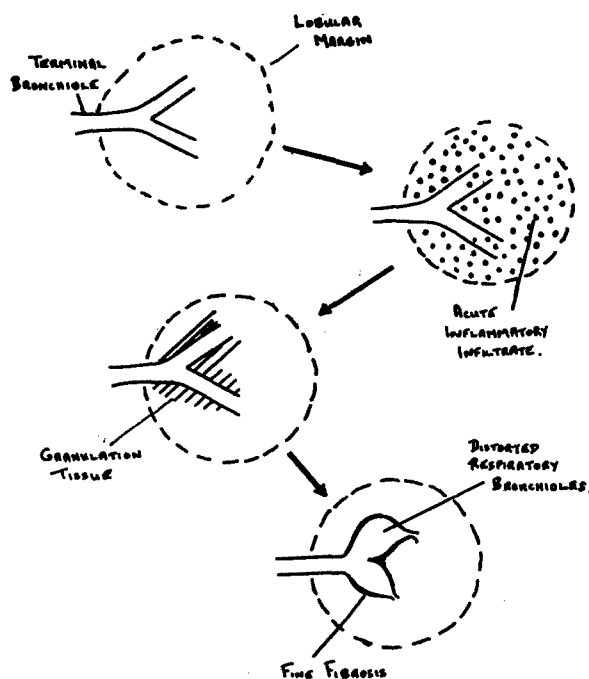


Figure 1. Diagram showing postulated sequence of events after multiple exposure to cadmium aerosol.

Damage Induced by Single Exposure of CdCl_2 Exposure

The site of the initial lesion in this model may well be obscured because of the multiple exposures as a result of which acute injury is presumably superimposed on healing injuries of differing age. For this reason the remaining studies have been carried out on male rats given a single two-hour exposure to

0.1% CdCl_2 in physiologic saline. This yields a consistently reproducible injury. Rats were exposed to 0.1% CdCl_2 in physiologic saline and killed by barbiturate overdosage in groups of six at one hour and 1, 2, 4, 7 and 10 days afterwards. Control groups consisted of groups of six saline-exposed animals killed at the same time intervals and also 35 rats not exposed to any form of aerosol. Both lungs were weighed and the left lung was used to determine the dry weight-wet weight ratio by drying to a constant weight in a vacuum oven. The right upper, middle and lower lobes were used to determine the enzymatic activities of lactic, malic and isocitric dehydrogenases (Colwick and Kaplan, 1957 and 1962); glucose-6-phosphate dehydrogenase (Ball and Jungus, 1963); RNA and DNA content (Munroe and Fleck, 1966; Ceriotti, 1952); and total extractable lipid (Folch et al., 1956). The protein concentration was determined by the biuret method. An additional number of rats, unexposed, saline and CdCl_2 exposed, were used for morphologic studies. The lungs were fixed by inflation with Karnovsky's glutaraldehyde solution, post-fixed in osmic acid, and processed for light and electron microscopy.

The lung weight of CdCl_2 exposed rats increased to a peak value at four days when it was double the lung weight in unexposed and saline aerosol exposed animals. These values are shown in Figure 2a as the ratio of lung weight to body weight. The wet weight of CdCl_2 exposed animals showed a significant increase ($p < .001$) at one day, but returned to control values by the second day. However, at ten days the dry weight of CdCl_2 exposed animals was significantly increased above the two control groups (Figure 2b).

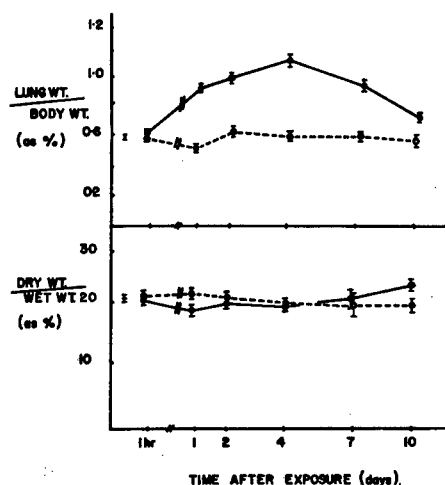


Figure 2. Effect of exposure to cadmium on rat lung weight.

Cadmium exposed (●---●)
Saline exposed (○---○)

Single points at left are means of unexposed rats over 10 day period. Each point represents mean value \pm standard error.

- Lung weight expressed as a percentage of body weight.
- Dry weight of lungs expressed as a percentage of wet weight.

Morphologic Changes

Little change is evident by one hour, but by one day there is an obvious increase in the number of cells throughout the lung. Although widespread, the change is multifocal rather than diffuse, some areas of lung being indistinguishable from control lungs (saline-aerosol treated or untreated), whereas adjacent areas show a marked cellular increase. In these early experiments it appears that the cell increase occurs near bronchioles, but this interpretation has yet to be confirmed by examination of serial sections. Thick (1 micron) sections show that the cellular areas are lined by cells with large nuclei and swollen cytoplasm which project into the alveolar lumen. Ultrastructurally the protuberant cells appear to be mainly Type II cells which have the usual content of osmiophilic inclusions (Figure 3). These cells show a variety of changes which probably represent nonspecific damage which include swelling of mitochondria with rarefaction of their ground substance, dilatation of rough and smooth endoplasmic reticulum, increase in vacuoles in epithelial and endothelial cells, and the appearance of neutral lipid droplets in the cell cytoplasm (Figure 4).



Figure 3. Rat lung one day after 0.1% CdCl_2 aerosol exposure. Alveolus showing increase of Type II cells of which there are six in the thickened wall (arrows). X4000.
(All electron microscopy photographs are stained with lead citrate and uranyl acetate.)

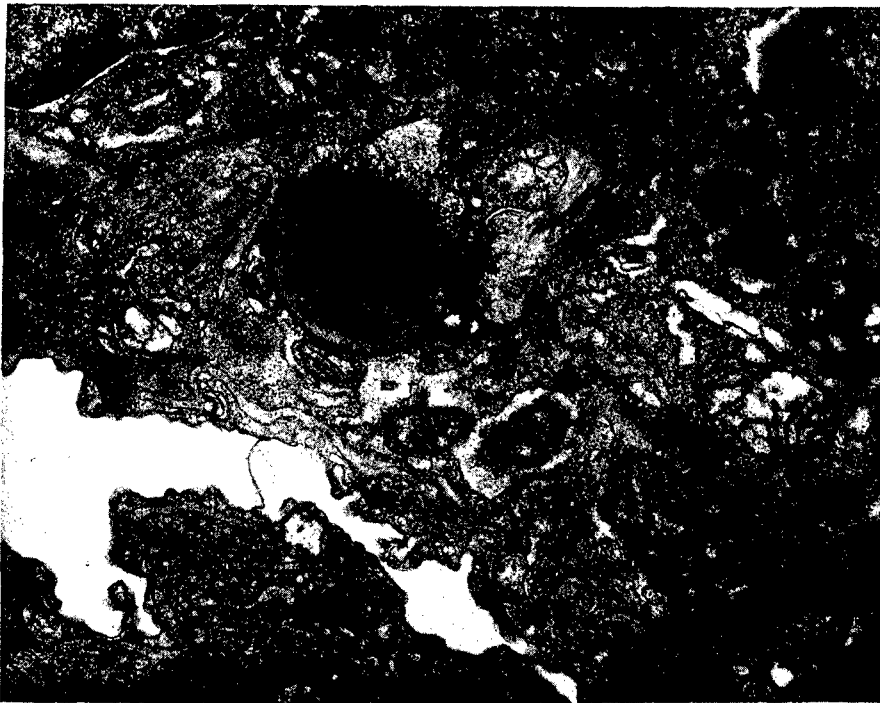


Figure 4. Rat lung one day after CdCl_2 aerosol. Some mitochondria are swollen with disturbance of cristae. Excessive epithelial cell vacuolization is seen. A large intracytoplasmic neutral lipid droplet is present. X15,000.

By the second day after exposure similar changes are seen, although they are more severe. In particular, distension of organelles and endoplasmic reticulum is prominent and a space is seen between the nuclear membrane and the adjoining cytoplasm which is electron dense. Additionally, in Type II cells the osmiophilic bodies become larger with more finely structured lamellae, while the microvilli are either lost or diminished in number (Figure 5). The swollen appearance of some of the cells bulging into the alveolar lumen is due in part to the formation of subendothelial blebs in capillaries or to cytoplasmic edema and swelling in Type I alveolar cells as shown in Figure 6.



Figure 5. Type II cell two days after cadmium exposure showing dilated endoplasmic reticulum, cytoplasmic vacuolization, large osmiophilic bodies, prominent nuclear-cytoplasmic gap. Mitochondria are swollen with splayed cristae. There are diminished numbers of microvilli. X3,000.



Figure 6. Two days after CdCl_2 . Alveolus showing subendothelial bleb (B) and edematous swelling of a Type I cell (T1). The granular contents of each resembles that of plasma within the capillaries (C). X5,500.

Another striking feature from two days onwards is the marked increase of free cells within the alveolar lumen. In areas showing little cellular increase in the airway walls there are few alveolar cells and those present appear to be normal alveolar macrophages. The plasma membrane of these cells typically shows numerous thin, pseudopodal outpouchings and the cytoplasm contains relatively few phagosomes which are small. Organelles and cytoplasm are well preserved, although there is some dilatation of rough endoplasmic reticulum (Figure 7a). In contrast the cell-rich areas show numerous swollen, rounded cells which have few if any pseudopodal structures, the cytoplasm in most being packed with large vacuoles (Figure 7b). Some of the vacuoles probably contain neutral lipid, but others show a granular appearance of varying density the nature of which is conjectural. Organelles are compressed between the vacuoles, mitochondria are swollen and their cristal structure appears disrupted to a variable extent.

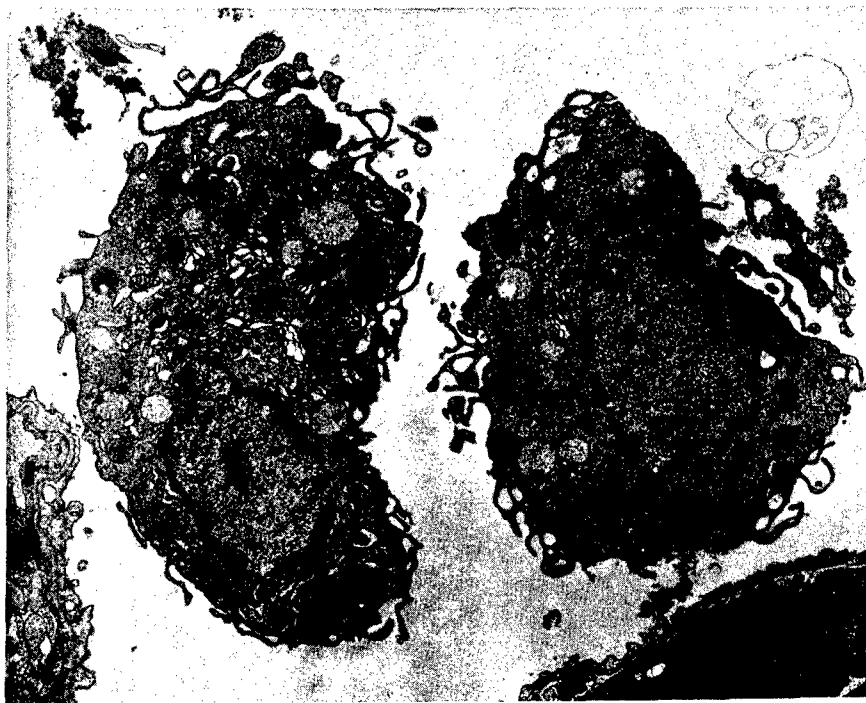


Figure 7a. Alveolar macrophages showing numerous fine pseudopods and scanty, small, cytoplasmic vacuoles. Mitochondria and other organelles show no evidence of damage. X4,000.



Figure 7b. Large, swollen cell with the cytoplasm stuffed with large vacuoles showing a variety of staining reactions. All the mitochondria are swollen and distorted. Microvilli are virtually absent. X4,000.

This pattern of cell increase reaches a peak by the fourth day and then returns by ten days towards that of the lungs in control animals. However, our initial observations suggest that an increase in the number of cells in the lung persists after the cadmium-induced injury and that alveolar walls are consequently thicker than in controls at ten days. This last observation remains to be confirmed. Another striking feature is the marked mitotic activity that occurs between one and four days after cadmium exposure, a feature which is readily appreciated in one micron thick sections. In order to study this mitotic activity, rats were exposed to CdCl_2 aerosol as above and then killed 1/2 hour, 1, 2 and 3 days later. Thirty minutes before killing each animal was given an intraperitoneal injection of $1.25\mu\text{Ci } ^3\text{H}$ thymidine/g. body weight. After inflation fixation with Karnovsky's glutaraldehyde-paraformaldehyde mixture, thick epon sections were processed for autoradiography. Weighed pieces of lung were also processed for scintillation spectrometry. Unexposed animals were used as controls. Autoradiographs showed that ^3H thymidine uptake in controls was low, averaging less than one labelled cell per alveolus (Figure 8a). In contrast cadmium-exposed animals showed increased uptake of ^3H thymidine at all observation times. This is

readily seen in the autoradiographs which show prominent labelling large, plump cells (? Type II cells) lining the alveolar walls, other cells in the interstitium and a certain proportion of the cells within the alveolar spaces. Figure 8b illustrates some of these features in tissue taken two days after CdCl_2 exposure where there are probably four or more labelled cells per alveolus. The uptake of ^3H thymidine was measured with scintillation spectrometry and Figure 9 shows that the peak occurs between one and two days postexposure, when the uptake is triple that in the unexposed controls.

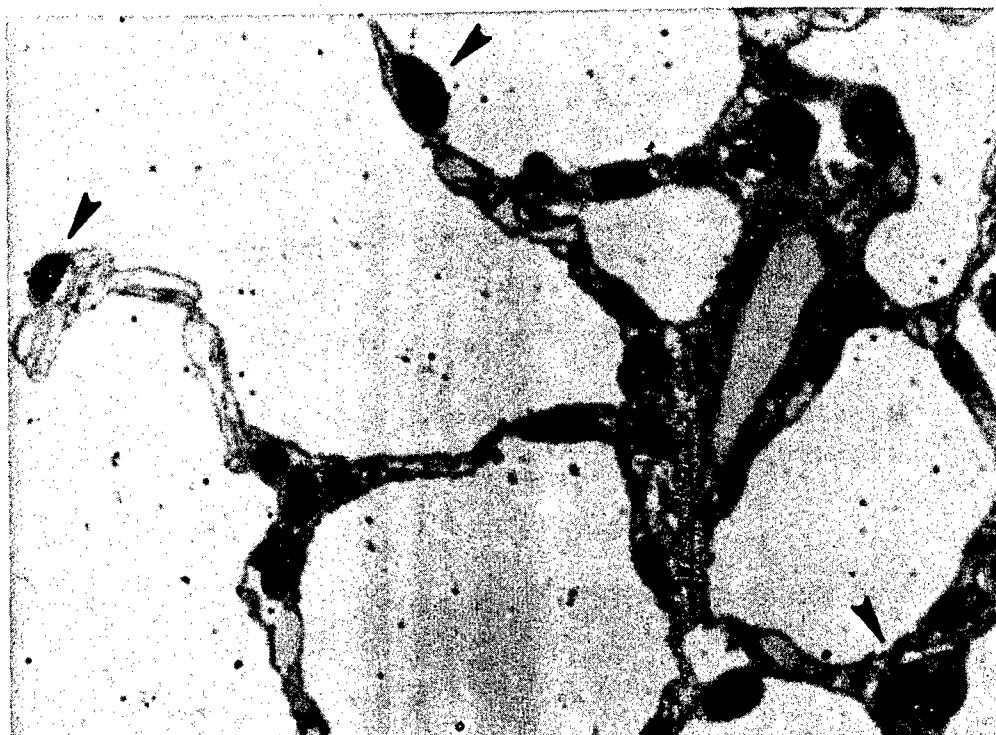


Figure 8a. Unexposed rat. Normal cellular pattern with uptake of ^3H thymidine in three cells (arrows), each in a separate alveolus. Methylene blue X400, oil.

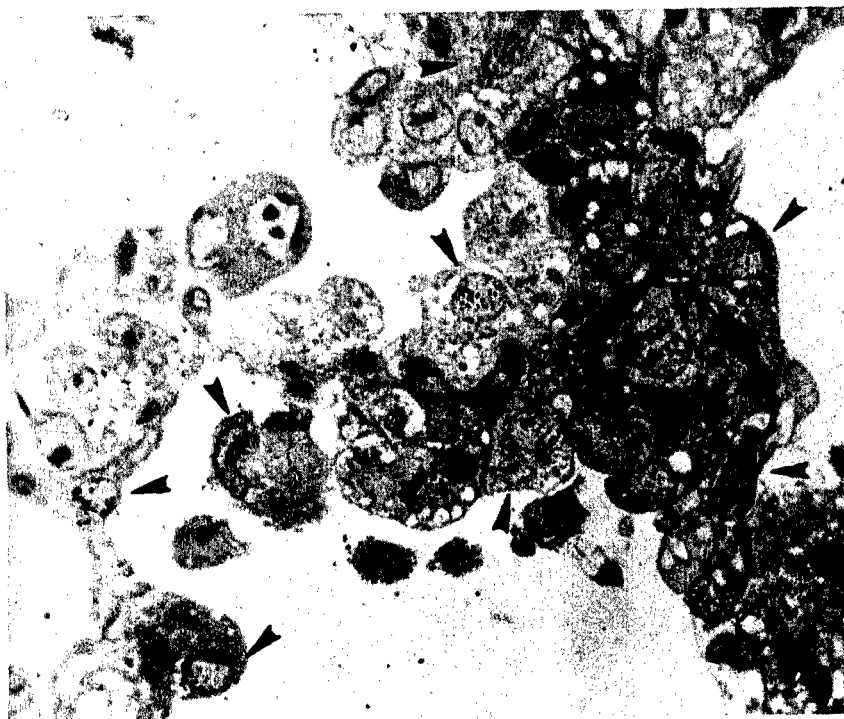


Figure 8b. Rat two days after cadmium exposure. Alveolar walls are plump due to increased cellularity and larger size of individual cells. ^3H thymidine uptake in nine cells (arrows) which in general resemble Type II cells. Methylene blue X400, oil.

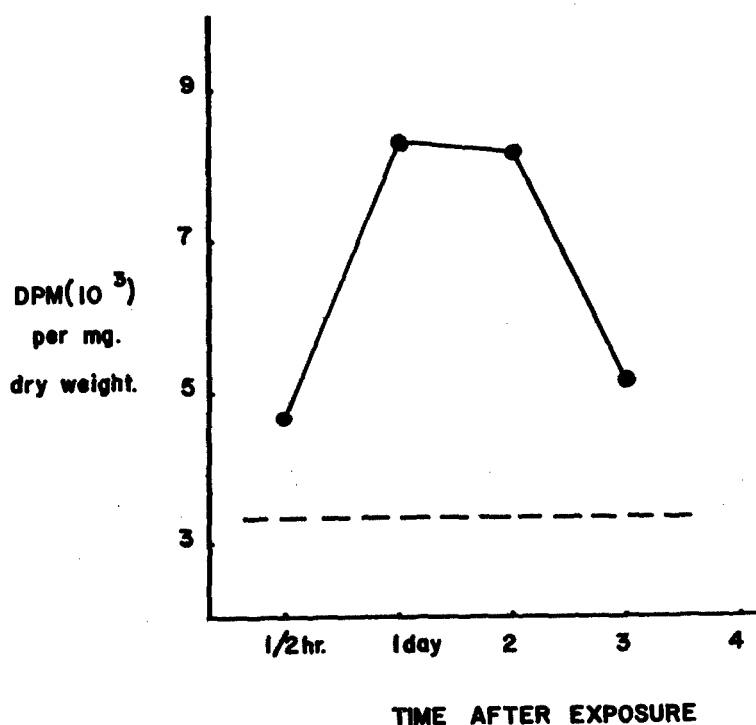


Figure 9. Uptake of ^3H thymidine after two-hour exposure to 0.1% CdCl_2 aerosol (●---●), expressed as disintegrations per minute X1,000 per mg dry weight. Mean value for unexposed control animals is shown by interrupted line (---).

Biochemical Changes

The increase in lung weight, the increased cellularity demonstrated by electron microscopy, and the transitory surge in ^3H thymidine uptake show that there is a numerical increase in lung cells and that many of the cells are probably larger in size (Figures 8a, 8b). This interpretation is borne out by the increase in extractable DNA and RNA in the lungs of cadmium-exposed rats (Figures 10a, 10b), an increase which peaks at four days at which time it is double control values ($p < .001$). By ten days control levels are regained. The pattern of RNA change is similar although the increase starts within the first day and is prolonged to four days, while the decline to control levels is more gradual than with DNA (Figure 10b). Interestingly the saline control group shows that an increase in extractable RNA occurs within the first two days, but returns to the normal range by four days. The reason for this is not clear.

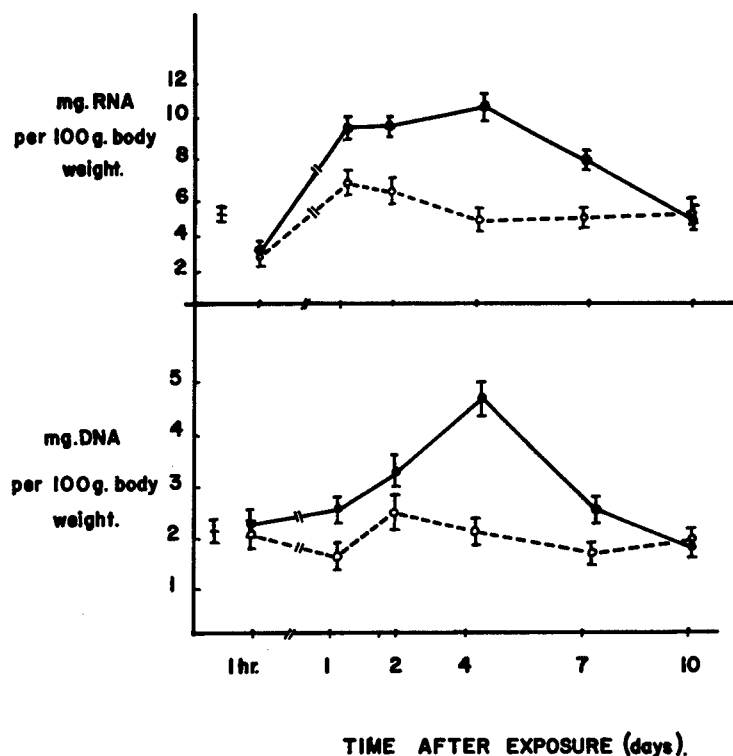


Figure 10. Nucleic acid content of rat lung expressed as mg/100g body weight following perchloric acid/potassium hydroxide extraction. CdCl_2 exposed (\bullet --- \bullet), saline exposed (\circ --- \circ). Single points at left are means of unexposed rats over 10 day period. Values represent mean \pm standard error.

a. RNA
b. DNA

These changes are paralleled by a 50 percent increase in glucose-6-phosphate dehydrogenase (G6PDH) which is maximum at four days ($p < .001$) (Figure 11). Increase in this enzyme activity presumably is coupled with increased pentose shunt activity in the formation of ribose required for nucleic acid synthesis. As the demand for RNA/DNA falls about the fourth day, so does G6PDH activity which returns to control levels at 7 days post-exposure. The alterations in lactic (LDH) and malic (MDH) dehydrogenase activities (Figures 12 and 13) suggest a more direct action of cadmium on these enzymes. A maximal increase in LDH activity of approximately 33% occurs at two days postexposure, coincident with the period of most rapid cellular proliferation. Here again, as the proliferative response of the lung subsides, LDH levels return to normal by seven to ten days.

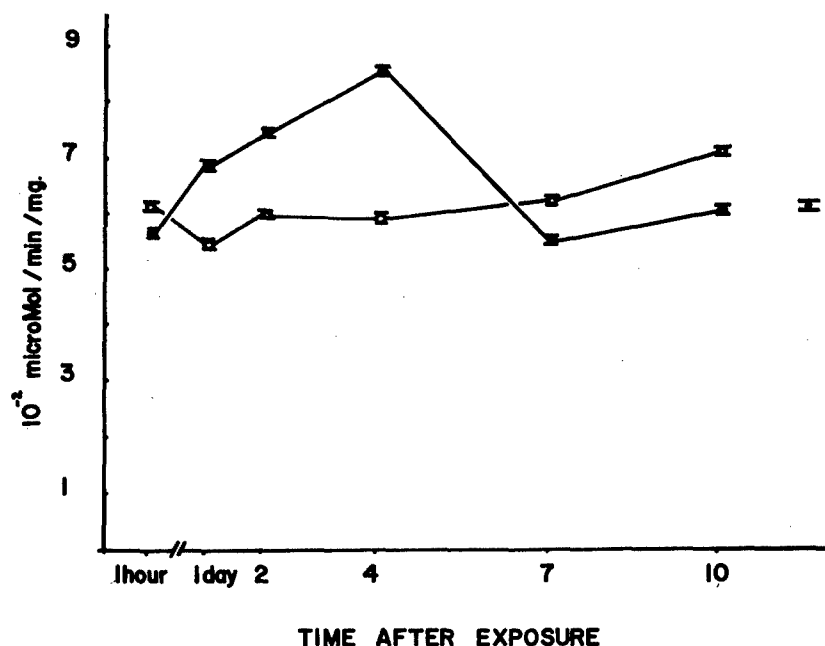


Figure 11. Alteration in glucose-6-phosphate dehydrogenase activity expressed as $\mu\text{M NADPH/min/mg}$ of protein. Cadmium exposed (●---●), saline exposed (o---o). Single point values represent \pm standard error. At right is mean of unexposed rats over 10-day period.

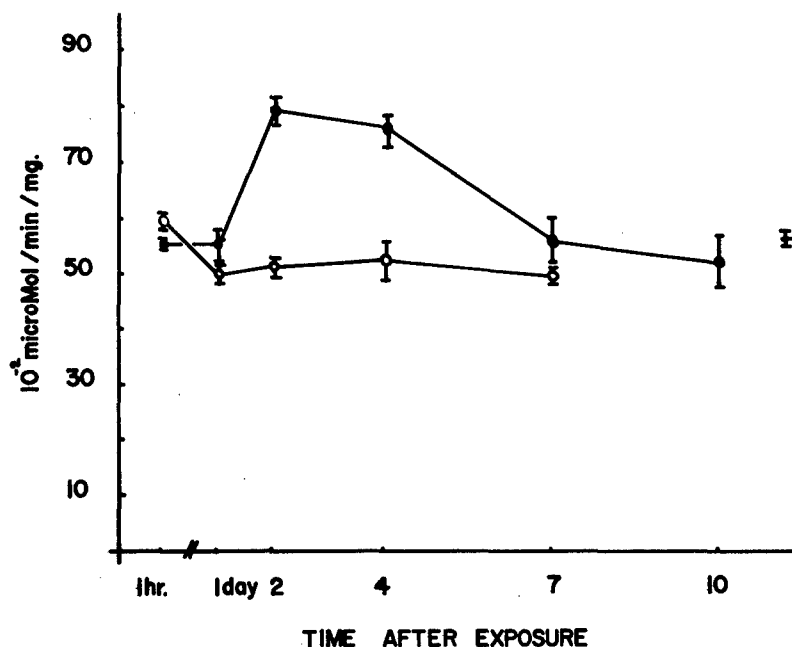


Figure 12. Lactic dehydrogenase activity μM NADH converted/min/mg of protein. Cadmium exposed (●---●), saline exposed (○---○). Single point values represent \pm standard error. At right is mean of unexposed rats over 10-day period.

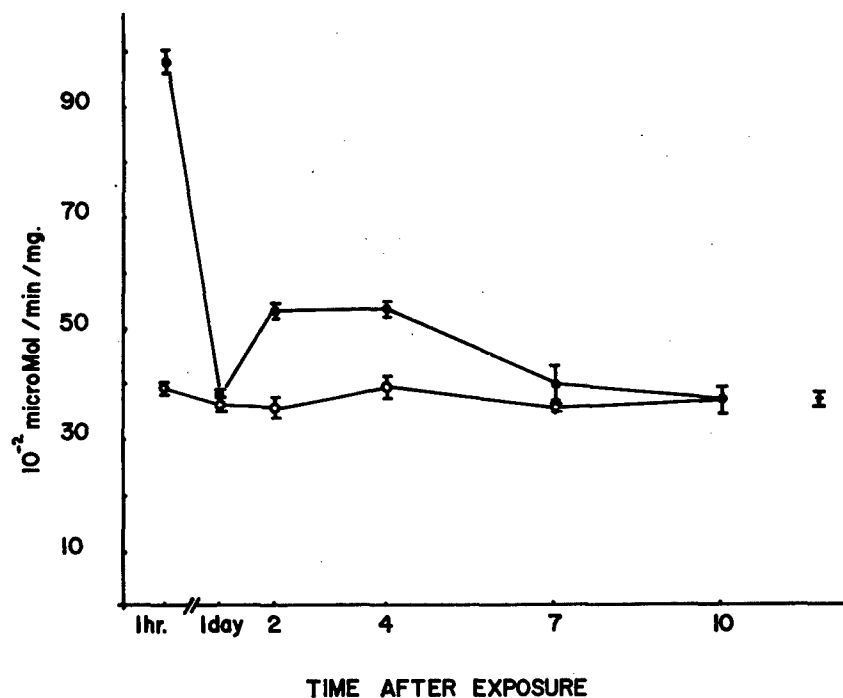


Figure 13. Changes in malic dehydrogenase activity expressed as μM NADH converted/min/mg of protein. Cadmium exposed (●---●), saline exposed (○---○). Single point values represent \pm standard error. At right is mean of unexposed rats over 10-day period.

Malic dehydrogenase shows the most marked response to cadmium aerosol exposure (Figure 13). Within one hour following exposure to cadmium, MDH activity has risen to a level 2.5 times that shown in control animals. This stimulation in activity is short lived, however, returning to control levels by one day. A biphasic response is seen with MDH as the period following exposure lengthens in that there is a second increase in activity of the enzyme to a value of about 37% above controls. A plateau exists at this level of activity for the period of two to four days postexposure, at which time a gradual decline in activity to normal takes place by the tenth day following cadmium aerosol exposure.

DISCUSSION

These findings show that a single exposure to cadmium aerosol induces widespread damage which is multifocal rather than uniform involvement of the entire lung. The structural changes are associated with a wave of cellular proliferation, as demonstrated by electron microscopy, autoradiography and scintillation spectrometry, with the peak activity occurring between 2-1/2 and 3-1/2 days after the insult. It appears that the Type II cell is the major proliferative component, although labelling experiments are being repeated to confirm this interpretation. If confirmed, this evidence will further support the contention that the Type II cell is the major reserve cell in certain, if not most, forms of lung damage as has been shown with injury produced by nitrogen dioxide (NO₂) (Evans et al., 1973); oxygen (Kapanci et al., 1969; Adamson and Bowden, 1974); and ozone (Stephens et al., 1974). It was observed that although cell division increased after NO₂, this was not followed by an increase in the cell content of the lung (Evans et al., 1973), a finding which is surprising. The results from the present study do not agree with this conclusion because the DNA content doubled by the fourth day after exposure before declining to control levels at the tenth day (Figure 10b). This indicates that cadmium induces an increase in lung cell content although, admittedly, it appears to be a transitory phenomenon.

Most of the cellular increase is undoubtedly related to intramural cell proliferation. However, the alveolar spaces also contain a large number of cells between the 2nd and 4th days after exposure. These alveolar cells show few if any pseudopodal cytoplasmic protrusions and have increased volume due to packing of the cytoplasm by vacuoles which are probably filled with phagocytosed material. These cells are similar to those seen after ozone damage which were thought to be alveolar macrophages (Plopper et al., 1973). It is not clear from the present experiments whether these free cells are alveolar macrophages showing evidence of recent phagocytic activity or whether they were damaged cells (? Type II cells) shed from the airway walls. It is significant that alveolar cell increase occurred mainly in areas showing evidence of mural cell proliferation, whereas

intervening "undamaged" alveolar spaces contained few cells and these had the ultrastructural appearance of normal alveolar macrophages. Irrespective of their precise origin, these free cells would seem to indicate sites of lung injury.

Accompanying these changes is a significant elevation of glucose-6-phosphate dehydrogenase activity which can be related to the increased need for ribose sugars in the synthesis of nucleic acids. The pattern of the elevation coincides well with the uptake of tritiated thymidine. The raised levels of lactic dehydrogenase suggest that there may be increased use of anaerobic pathways. It has recently been shown that when isolated mitochondria from sheep alveolar macrophages (Mustafa and Cross, 1973) are incubated with cadmium-containing media, there is profound depression of oxidative phosphorylation and also of the electron transfer pathway which would increase the need for anaerobic metabolism. Mustafa showed that CD^{++} depressed dehydrogenase activity so that the pattern of delayed increase seen in our experiments could represent a rebound phenomenon following initial depression. This explanation does not readily account for the very high level of malic dehydrogenase activity. We think that the MDH activity at one hour may be due to leakage of enzyme from mitochondria, possibly following damage to the membrane by nonspecific binding (Jacobs et al., 1956).

In summary, exposure of rats to cadmium aerosol, of an amount roughly equivalent to one pack of cigarettes in man, produces a marked wave of cell proliferation and a delayed wave of increased dehydrogenase activity. The mechanisms inducing these changes are presently unclear.

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OPEN FORUM

DR. TYLER (University of California, Davis): I thought perhaps we could start off our discussion with a discussion of the paper presented by Dr. Robin. I think that what has been presented today was the reaction of the lung to several injurious agents, specifically ozone and cadmium. These reactions were quite similar to those of other injurious agents. I wonder, Dr. Robin, if you might speculate about the mechanisms or limited ways which organs have of reacting to chemical agents.

DR. ROBIN (Stanford University): First of all, let me say that the outline I presented was very broad and the picture that I painted was painted with very broad strokes. I hope everyone will forgive me for this artistic license. It's very easy when you go from broad outlines to specific areas to be very oversimplified in one's approach. It's like learning to speak a new language, and even though you can draw diagrams of impairment of energy metabolism on the blackboard, that's a long way from the laborious and complicated work involved in proving how energy metabolism is affected and what the mechanisms are, ultimately on a molecular basis. So I suppose I really should apologize to the audience. It really isn't as simple as the model suggests that it might be. It seems to me that the papers presented in this program fit in very well with this broad concept because it's apparently relatively easy to get structural and ultrastructural data. How to ultimately measure disorders in function is much more difficult and that, of course, will require much more intensive studies on cell function. Therefore, the attempts that have been made to look at enzyme activity or at ultratransport are still in their infancy and lack a good deal of penetration. It seems to me that it's relatively clear that one of the things that both agents described today is to alter cell and subcell membranes, and that as a result of this alteration, there is a development of intracellular edema, some cells being more affected than others. I suppose that one could, with some degree of precision, work out the mechanism by which ozone, for example, alters membrane integrity leading to intracellular edema and then leading to the other changes that occur in mitochondria as a result of intracellular and subcellular edema to then produce abnormalities of energy metabolism.

DR. CROCKER (University of California, Irvine): I'm impressed by Dr. Robin's interest in trying to carry over to toxicology a pharmacologic principle involved in the absorption, distribution and excretion or elimination of the toxic material. I think that this is a challenge that's rather hard to meet with some of those toxic materials that are, in their own right, very simple molecules that have a very short biological half-life or that have a way of merging into a pool from which it's hard to recover them. The homeostasis by which the body receives an input of high energy is a hard thing to qualify in terms of the amount put in and the amount eliminated. But there's a new concept, I think, that I'd like you to talk to. And that is that with toxic agents, you're dealing

with a capability of the body to sustain the injury and to undergo a kind of repair which, up to a limit, is physiologic and beyond that limit is pathologic. There appears to be a steady state or some limiting state of response that is incurred in a toxic injury that may not be incurred in the ordinary delivery of pharmacologic agents. I think the integration of these two factors, that is how to tie the input distribution and its outflow to an injury that is essentially a high energy type of damage and the matter of equating or learning what the physiologic response ceiling is above which there is a pathologic event are two special challenges that we're facing in our efforts to work with high energy inhalants.

DR. ROBIN: I certainly agree, Dr. Crocker, that the technical problems with many toxic agents are considerably more formidable than with many pharmacologic agents. I would, however, like to suggest that one of the things that's occurred with the use of pharmacokinetics are enormous numbers of surprises. Drugs that were felt to be very inactive or to have very wide biological scatter from the standpoint of effects were found not to be necessarily inactive and the scatter was in the minds of the observer. That is, once measurements were made of kinetics, it became perfectly clear why there was scatter or why one couldn't come up with these data. Since I believe that one can technically deal with the problems of delivery of toxic agents in this kind of format, it seems to me that such data is absolutely required. It may be that a substantial amount of it will not prove to be useful in ultimately elucidating the mechanism of action but don't knock it until you've tried it. You really can't tell. I think, therefore, that it really isn't scientifically acceptable at this point in time to deal with simply output or blood levels. The second point that I'd like to make is to agree with you that there are many agents in which the impact of the agent comes long after the agent has left the body and indeed may be a response to the metabolites rather than having anything to do with the primary effects of the agents. I think that a scheme does convey difficulties, then, which looks at cellular abnormalities. This approach is much more likely to pick those effects up than screening approaches which simply deal with gross changes affecting the whole animal. The third thing that I'd like to say, which perhaps I didn't make clear, is that just as the toxicologist of the 1950's and 1960's and maybe even back to the 1940's would subject animals to a given toxic agent and then look at the blood and then count red cells and enumerate the hemoglobin and hematocrit and look at the urine and see whether there were some proteins in it and look at the blood and estimate the amount of sodium and potassium and so on, it seems to me that it would make much more sense for toxicologists of the 70's and 80's to look at cells and see how they conduct energy metabolism and transport and so on as a much more meaningful elucidation of the net impact of toxic agents of biological systems, even intact ones.

DR. HODGE (University of California, San Francisco): I hope you won't misunderstand, Dr. Robin, I've been keenly conscious of the point that you brought out ever since I really got into toxicology. I believe that probably you

would find most toxicologists sharing your opinion. One of the frustrating and exasperating aspects of toxicology is to find that there is a little bit of information here and a little bit there. It seems like an electron micrograph with structures all over the place and no known unifying connection. So what I was smiling about just now was that I was dreaming about an ideal toxicology laboratory. Maybe this is 1984. I started down the hall and I was pleased to see that there was a door that said "Structure" and it said Weibel on it. Just a little further, there was a door that said "Transport" and I found Dr. Tosteson in there. Next I saw "Metabolism" and there was Dr. Tecwyn Williams, and right beyond it, there was a door that said "Kinetics" and here was Dr. Steve Brody. The hall went way, way down and at the start of it, a sign said "The Laboratory for the Study of Ozone Toxicity." I'm not trying to poke fun at your idea, I'm sold completely on the importance of a unifying concept, but the problem of the toxicologist is that we have almost as much of a problem in dealing with a real identification and understanding of ozone toxicology as the biochemist and physiologist and pathologist do of understanding normal function.

DR. ROBIN: I'd like to say, Dr. Hodge, that obviously the universe is very difficult to understand except for a few exalted people, but the point is that there is a science which is now capable of being used in precisely this way. And might I give you an example from this meeting. I was very much impressed with Dr. Van Stee's presentation. But I'd like to say that it seems to me over the last 6 years, he's repeated exactly this sequence. He started out with an isolated observation that freon and halogenated hydrocarbons produce an increase in the oxygen content of the coronary sinus blood, and as he tried to explain that, he found himself looking at uncoupling and mitochondria. What I'm saying is that I would guess it took him 6 years to get to that point and I think it's possible now to get to that point much more rapidly by approaching it explicitly. I don't think you can have your lab with Dr. Tosteson and Dr. Williams and so on, but I certainly think that at your lab, you can have a cell physiology section which stands between the people who are making the most advanced contributions and between the classical toxicologist who can add that dimension to your studies. I personally think that's very practical and very sensible.

DR. DOST (Oregon University): Well, I detect a certain gentleness in the response to some of the comments that have been made by Dr. Robin and others. It has been some time since we did our toxicology by exposing a number of animals and counting the living ones and the dead ones and hoping they come up to the total we started out with. I would say that the sophistication in toxicology labs whether we're talking about pharmacokinetics or toxicokinetics, or studies of energy metabolism, or any other parameter group that you wish to consider, are certainly as advanced as they are in the field of pharmacology. We're not in the back woods and Dr. Hodge explained some of the difficulties that we have to face. Frequently, we don't even know what

compound we're dealing with as it's a little difficult to get out of that situation and into a situation where we're studying pure materials. The other point is that the toxicologist has a universal problem. He doesn't choose the compounds that he deals with. They are thrust upon him. That is one of the basic differences between the toxicologist and the pharmacologist. He's solving problems that have been given to him and not problems that he's chosen to attack.

DR. TYLER: Could I, as the chairman, comment very briefly on this aspect of it. Of course, in his daily work, Dr. Robin indeed, has many problems thrust upon him, because toxicologists have not identified problems and so he sees the human patient. Much as in the discussion of carcinogenesis yesterday, too many times a compound was found to be carcinogenic by keen clinical examination of human patients rather than by experimentation so that I think there are both points.

Dr. Neurath, I can see that monoprogramming indeed is very difficult and very tedious. Once you have developed the programs, are they applicable to a multitude of problems?

DR. NEURATH (Tufts New England Medical Center): Well, some portions are. Images have certain things in common. I took one example. We divide the scene visually very often into different objects by drawing or imagining boundaries. In fact, the eye is extremely well equipped to set a boundary even if there isn't one there as a physical reality. So, programs that extract boundaries can obviously be used in many areas. But, I think it's probably fair to say that because of a variety of images, programs tend to be rather specialized to particular aspects. Some things are very easily recognized by colors. If the differences that you look for are in color, programs that extract color information will be the important ones. Texture is a difficult thing to quantify and particularly to compute because it involves many things at once. A boundary sort of can be specified one point at a time but texture is how many dots and how big are they and how are they distributed and what is their shape. All that together makes up texture and there are all kinds of names that pathologists and microscopists use to describe texture; feathery, fuzzy, and a whole host of adjectives. There's no simple answer to that question. You get better at it as you go along. After having written many programs, it's easier to write another one to describe something else.

DR. TYLER: Could I ask, what is the speed of examination of a micrograph such as those micrographs of red cells? What is the time frame on the delivery of that information?

DR. NEURATH: Let me give you a range. In the analysis to determine the differential white blood count, the aim is to count one cell in one second or less. That includes everything: finding it, scanning it, extracting

parameters and saying this is that kind of cell. At the moment in our chromosome analysis, to look at 46 chromosomes, to size what they are, to correct errors the computer makes, takes a little less than 20 minutes. If you take much longer, you can't afford to do it. These methods are not just limited by time. Your interest sort of lags and you can't afford a computer if it takes you all day to examine a single cell. Computer equipment is still improving at a very appreciable rate. I think the problems of what a person really looks for, many of the problems in the programming techniques are not the computer, but it's very hard to describe a structure. You can teach people to recognize things by showing them examples, and they say, "Oh, yes, this looks different," and then they sort of vaguely look for descriptive words but they learn quite quickly. If you show a person 3 images of one kind and then a variation, a sick cell or one that has been affected, they can pick that out. But to describe it in the kinds of terms of what the boundary length involved of what exactly the differences in color are, is very difficult. You can use a completely different stain and after a few examinations, you can recognize the same thing even though in one case it's blue and gray and the other blue and purple. That's extremely difficult to quantitate. Once you know what it is that you're really looking at and can put it down on paper in equations, then you can compute it. But if you can't, it's difficult.

DR. TYLER: I think it's obvious that biologists and , more specifically, morphologists, have to be able to describe to you how we do identify structures. Indeed, we do have to develop methods of looking at structures which are suitable for your equipment so as to permit your equipment to operate at its highest efficiency and enhance our own efforts. I think that research in specimen preparation for these automated systems is going to be very helpful. Perhaps the staining techniques which have been pleasing to the human eye are indeed not the best techniques for that instrument.

DR. GROTH (National Institute for Occupational Safety and Health): Dr. Phalen, you mentioned something about the physical properties of a particle being responsible for the lesion in the lung. Could you elaborate on that?

DR. PHALEN (Lovelace Foundation): Yes, I think the two materials that I mentioned were asbestos and quartz. I'm not an expert on either, but the theories of the mechanisms of toxicity for both of the materials, I believe, have involved at many times a particle shape, or particle size or particle surface character. Quartz is toxic in a particular form but other crystalline forms of the same material with the same chemical composition are not. Investigators have postulated that the physical shape of an asbestos particle contributes to its toxicity. Is that what you were after?

DR. GROTH: In other words, the shape of the particle determines its deposition site and retention?

DR. PHALEN: Not only that, but perhaps also the response of the organ. For example, the lung. Quartz does seem to be toxic to cells when they come in contact with its surface for reasons probably related to its crystalline structure.

DR. GROTH: You don't think this is more related to the solubility of the quartz or silicates?

DR. PHALEN: I don't think so. Again, because the different crystalline forms have vastly different degrees of toxicity. So I don't think that we have a chemical problem here. You have a comment on this?

DR. GROTH: Yes, if I might. I think that Dr. Allison in England has clearly shown that the interaction between quartz and macrophages is related to hydrogen bonding at the surface of the crystal and that is basically a chemical reaction and not a physical reaction. Indeed, if you take crystals which cannot be hydrogen bonded, then you don't get the inflammatory response. The same model, interestingly enough, applies to gout and pseudogout where in inflammatory joint disease, you have the uptake of crystals which are capable of being hydrogen bonded within macrophages and the crystal then is in a sense needling the joint surface lining, but its needling is not because it sticks into it in a physical sense but because it evokes chemical reaction which is relatively well understood.

DR. PHALEN: Does this reaction imply that the crystal is dissolving or that it has a structural relationship that sets up a reaction?

DR. GROTH: Well, I suppose in ultimate physical chemical terms you couldn't get hydrogen bonding unless you had some degree of solution. Now, the solubility coefficient may be extraordinarily low but it's still large enough to permit some kind of hydrogen bonding.

DR. PHALEN: Okay, I'll defer to your expertise on that. I think that Werner Sturber in Germany has shown also that the response to quartz is quite great compared to the amount of material that could be expected to dissolve over the time period or enter actually into the body. I think that's all of the information that I have on it.

DR. GROTH: I guess all I'm saying is I think that firm biochemical evidence exists that even if you make quartz crystals round so that they weren't pointed and you changed their size, they still would be taken up by macrophages, be hydrogen bonded, evoke lysosomal rupture in an inflammatory response.

DR. PHALEN: I won't argue with that.

DR. CULVER (University of California, Irvine): I wonder whether or not zinc fume might be considered to be an example of the effect of physical

characteristics upon a biological system in that freshly generated zinc fume is far more biologically active than aged zinc fume.

DR. PHALEN: Possibly. It sounds like you know a little bit more about that than I do. I'm basically a physicist. I'm not familiar with the toxic mechanisms of zinc. I know that aging of particles, fumes would certainly be included, often involves a change in the structure of the material at its surface in that there are surface tension forces that break down and distort the normal crystalline structure. This sort of aging occurs for sure in aqueous suspensions and most probably in air, but I can't comment really on the mechanisms of toxicity of zinc.

DR. GROTH: I'd like to ask a question of Dr. Hayes. Did you see any metaplasia in the lungs and does the cadmium localize in the collagen? Do you know anything about that?

DR. HAYES (Mallory Institute of Pathology): You mean one exposure or recurrent exposures?

DR. GROTH: In your experiments.

DR. HAYES: No, the injury seems to be relatively acute, limited and seems as far as we can tell to be completely healed. I don't know where the cadmium goes, but it's in the lungs somewhere. It could well be bound into hemoglobin and taken away by that mechanism. It could be interstitially bound to metallothioneine or some other mechanism. I don't know anyone who's worked on this matter. One of the things we would like to do is to trace the cadmium into the tissues, but basically, I have no information on this.

DR. CROCKER: I think that maybe some of the workers that have done the ozone work have done some of the cellular kinetic measurements that were described for cadmium. Would you care to describe any of those?

DR. SCHWARTZ (University of California, Davis): Yes, cellular kinetics studies have been done by the Evans group at Stanford and they have demonstrated the increased turnover rate or increased production of Type 2 cells by tritiated labelling. That's about all I'm really familiar with.

DR. CROCKER: Right, I'm familiar with that study. I was thinking more of the kind of gross studies, whole lung preparations that made it possible to make some quantitative estimations and time scale of response by means other than the rather difficult sampling process of electron microscopic autoradiography which was involved in the Evans study.

DR. SCHWARTZ: I'm really not familiar with that work. Perhaps Dr. Dungworth and Dr. Tyler would be.

DR. DUNGWORTH (University of California, Davis): We did some work on the use of light microscopy on thin sections. The work of Evans' group was mostly involved with changes occurring within the first 72 hours after NO_2 or ozone exposure. Their results were somewhat similar to the ones shown by Dr. Hayes as far as the dynamics were concerned. I think the amount was maybe less because the degree of insult they gave was not as great. As you well know, Evans by means of the labelling method showed what was the first reasonably conclusive evidence that the Type 2 cells changed to Type 1 during repair of one of the daughter cells, and that finding was later confirmed by Bodin. That was, I think, the other main part of his study.

DR. CROCKER: The thing that I would just like to finish with on this point is that in the measurements that were made by Dr. Hayes, the insult had to be fairly heavy. The measurements seen in morphologic terms were more extensive than those seen in the ozone study. The ozone study measured effects at concentrations that are in fact in the realistic level of human exposure whereas the cadmium study was, in a sense, a probing first level of experimental observations. It will go down to lower levels, I'm sure, in the future. The point is at the time when you have to use the most sensitive method, it may be possible that a mass measure of thymidine incorporation by the whole lung, or of glucose-6-phosphate dehydrogenase activity or any of the other measurements that were suitable for whole lung studies in a fairly heavy insult might fail to be sufficient in a study that was very limited in toxic effect. Thus, the technique used of scanning electron microscopy and morphology in general may prove to have been the most sensitive to the least possible damage at levels of human exposure.

DR. NORDBERG (University of North Carolina): I'd like to elaborate a little on the point of the doses of cadmium especially since Dr. Hayes mentioned his results in relation to the effect of smoking. He mentioned that the doses he had used were similar to those inhaled when smoking a pack of cigarettes. I was not quite aware of what dose levels you actually used. Could you elaborate a little on this in relation to smoking? As far as I know, it has not been reported in industrial exposures that emphysema occurs at levels below something like 100 micrograms of cadmium per cubic meter of air. Maybe you can give some more information on this point.

DR. HAYES: We don't have a specific dose level. This was calculated in retrospect from the amount of accumulated cadmium in the lung measured after the exposure. The actual dose levels we are working on now. We have a new chamber. We are going to use particles of a standard size, presently we are starting off with 5 micron diameter particles and by controlling air flow and concentration of the solution, we will then establish a specific dose. But even so, this is a little difficult to estimate because it's not until we know how much of the metal goes into the lung that it makes any sense. I presume that the metal retained in the lung is doing the damage rather than the stuff that goes in and comes out. I don't think it's a hit and run rate, as it were.

CLOSING REMARKS

DR. CULVER (University of California, Irvine): In looking back over the past three days of the conference on subjects ranging from ecological systems to subcellular function, I've been impressed with the scope of our task and our interest. The field of environmental toxicology is as vast as all of the sciences that contribute to its understanding. Dr. Hodge's desire for a laboratory composed of specialists in the fields of structure, energy, transport mechanisms is the only logical approach to solving the problems with which we are confronted. There is one other element that is needed in the operation of such a laboratory and that is a system of communication that will serve all of the people who live in those little rooms down that long hall. It is my observation that much of what we've been doing here the past few days is working on the development of a common language. We've been sharing with each other our own symbology, our own specialized language and we have been in the process of building this common language and this work that we have to do is going to require a team of specialists who can all talk together. The talking together that has been done these past three days has been very impressive. As I look back over the three days, I find that I have taken with me a few pieces of that language that will serve me as bridges to the disciplines that are represented here today. I hope each of you leaves this meeting with a feeling that you, too, have gained something from the presentations and that you will come back and join us again next year. I wish to thank the Aerospace Medical Research Laboratory, the participants, the attendees and the staff who have done such a wonderful job in making this a step forward in the progress of environmental toxicology. Thank you very much.